EXHIBIT 1

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Pamela E. Bechtel, declare as follows:

1. I am a co-inventor of the subject matter of the above-identified patent application.

2. I received my Bachelor of Science degree in 1993 from Pennsylvania State University. In 1998, I was awarded the degree of Doctor of Philosophy in Pharmacology and Toxicology from the University of Maryland.

3. Exhibit A presents a true and correct copy of my doctoral dissertation entitled: PROLIFERATING CELL NUCLEAR ANTIGEN IN MALIGNANCY, submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998.

4. My doctoral dissertation (Exhibit A) describes and evidences the presence of a cancer specific isoform of proliferating cell nuclear antigen (csPCNA) in various malignant cell lines, including malignant prostate cells, colon cells, brain and cervical cells, and leukemia cells.

5. Figure 1 (page 101 of Exhibit A), the Legend for Figure 1 (page 99 of Exhibit A), and the RESULTS for PCNA in Malignant Prostate Cells (page 88 of Exhibit A) evidence the presence of the csPCNA isoform in malignant prostate cells.

6. Figure 2 (page 102 of Exhibit A), the Legend for Figure 2 (page 99 of Exhibit A), and the RESULTS for PCNA in Malignant Colon Cells

(page 88 of Exhibit A) evidence the presence of the csPCNA isoform in malignant colon cells.

7. Figure 3 (page 103 of Exhibit A), the Legend for Figure 3 (page 99 of Exhibit A), and the RESULTS for PCNA in Malignant Brain and Cervical Cells (page 88 of Exhibit A) evidence the presence of the csPCNA isoform in malignant brain and cervical cells.

8. Figure 4 (page 104 of Exhibit A), the Legend for Figure 4 (pages 99-100 of Exhibit A), and the RESULTS for PCNA in Leukemia Cells (page 89 of Exhibit A) evidence the presence of the csPCNA isoform in Leukemia cells.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 11/21/06

Pamela E. Bechtel

1708500

EXHIBIT A

1. Pamela E. Bechtel's doctoral dissertation entitled: PROLIFERATING CELL NUCLEAR ANTIGEN IN MALIGNANCY, submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998.

APPROVAL SHEET

Title of Dissertation: Proliferating Cell Nuclear Antigen in Malignancy

Name of Candidate: Pamela Elaine Bechtel

Doctor of Philosophy, 1998

Dissertation and Abstract Approved by: _____

Robert J. Hickey, Ph.D.
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Date Approved: May 8th, 1998

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Pamela Bechtel, Robert Hickey, Jennifer Weeks Sekowski, Lauren Schnaper, Brian J. Long, Carla Rodriguez-Valenzuela, Ni Lui and Linda Malkas. Malignant Breast Cells Contain a Unique Form of PCNA. Cancer Research (in press, 1998).

Jennifer Sekowski, Linda Malkas, Lauren Schnaper, Pamela Bechtel, Brain Long and Robert Hickey. Human breast cancer cells contain an error-prone DNA synthetic apparatus. Cancer Research (in press, 1998).

Poster presentations:

The Cloning and Functional Characterization of the Human Dopamine Transporter. Pamela Bechtel, Hong Dai and Robert Hickey. Presented at The Molecular and Cell Biology Symposium on Signal Transduction at the University of Maryland at Baltimore in Baltimore, MD, April 1995.

The Identification of Structural Alterations in the DNA Synthetic Apparatus of Human Breast Cancer Cells. Pamela Bechtel, Jennifer Coll, Robert Hickey and Linda Malkas. Presented at the annual American Association of Cancer Research meeting in Washington, DC, April 1996.

2D SDS PAGE Analysis of the DNA Synthetic Machinery from Malignant and Normal Breast Epithelial Cells. Pamela Bechtel, Jennifer Weeks Sekowski, Robert Hickey and Linda Malkas. Presented at the Histopathobiology of Neoplasm workshop sponsored by the American Academy of Cancer Research, Keystone, CO, July 1996.

Alterations in the Structure and Function of DNA Synthesome Components in Malignant Breast Cells. Jennifer Weeks Sekowski, Pamela Bechtel, Lauren Schnapper, Yuetong Wei, Robert Hickey, and Linda Malkas. Presented at the San Antonio Breast Cancer Symposium in San Antonio, TX, December 1996.

Fidelity of DNA Replication in Malignant and Nonmalignant Breast Cells. Jennifer Sekowski, Pamela Bechtel, Linda Malkas and Robert Hickey. Presented at the annual American Association of Cancer Research meeting, San Diego, CA, April 1997.

The Identification of Protein Alterations of the DNA Synthesome Components in Breast Cancer Cells. Pamela Bechtel, Robert J. Hickey and Linda H. Malkas. Presented at the Graduate Student Research Day, University of Maryland at Baltimore in Baltimore, MD, April 1997.

Malignant Breast Cells contain a Unique Form of PCNA. Lori Croisitierre, Pamela Bechtel, Robert Hickey, Brian Long, Robert Obar, Bryn Watkins, Lauren Schnaper, and Linda Malkas. Presented at the annual American Association of Cancer Research meeting, New Orleans, LA, March 1998.

Inhibition of the Human Cell DNA Synthesome through the Interactions of p21 and PCNA. Derek Hoelz, Pamela Bechtel, Robert Freund, Robert Hickey, and Linda Malkas. Presented at the annual American Association of Cancer Research meeting, New Orleans, LA, March 1998.

MCF-7 Breast Cancer Cell DNA Cynthesome as a Regulatory Center

for Estrogen Induced Cellular Proliferation. Carla Rodriguez-Valenzuela, Pamela Bechtel, Brian Long, Phillip Wills, Robert Hickey, Angela Brodie, and Linda Malkas. Presented at the annual American Association of Cancer Research meeting, New Orleans, LA, March 1998.

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ABSTRACT

Title of Dissertation: Proliferating Cell Nuclear Antigen in Malignancy

Pamela Elaine Bechtel Doctor of Philosophy, 1998

Dissertation Directed by: Robert J. Hickey, Ph.D. Research Assistant Professor, Dept. of Pharmacology and Experimental Therapeutics and Greenebaum Cancer Center; Programs in Toxicology and Molecular and Cell Biology; University of Maryland

The development of a malignancy is a multistep process that is not clearly understood. Nonmalignant cells undergo a transformation process resulting in aberrantly proliferating malignant cells suggesting that the replication machinery of malignant cells is altered. In this study the DNA replication apparatus (the DNA synthesome) was examined to identify proteins altered in malignant cells. Analysis of malignant and nonmalignant cells by two dimensional gel electrophoresis (2D PAGE) demonstrated that many malignant cell types contain a unique, acidic form of proliferating cell nuclear antigen (PCNA). This protein is a 36 kD nuclear protein required for DNA replication and DNA repair. The unique form of PCNA was found in malignant breast, prostate, colon, cervical, brain and

leukemia cell lines and in malignant human breast tumors and chronic myelogenous leukemia specimens. Serum collected from a breast cancer patient was analyzed and found to contain the cancer specific form of PCNA. Analysis of nonmalignant breast tissue and serum collected from cancer free individuals demonstrated that the cancer specific form of PCNA was not present. Further experiments were performed to characterize the unique form of PCNA. 2D PAGE analysis was performed on nonmalignant, transformed breast cell lines overexpressing the oncogenes c-myc (A1N4 myc) and SV40T (A1N4T). The cancer specific form of PCNA was present in these transformed cells. It was also determined that the acidic from of PCNA was not the result of growth stimulation or genetic mutation, suggesting that differential post-translational modification may be responsible. Although PCNA does undergo poly (ADP-ribosylation), 2D PAGE analysis demonstrated that the cancer specific form of PCNA was unmodified. The cancer specific form of PCNA appears to be a fundamental characteristic of malignancy and its role in tumorogenesis needs to be examined. These results suggest that epigenetic changes may contribute to the development of cancer.

Proliferating Cell Nuclear Antigen in Malignancy

by

Pamela Elaine Bechtel

Dissertation submitted to the Faculty of the Graduate School

of the University of Maryland in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

1998

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I would like to thank my thesis advisor Robert Hickey for terrific mentorship. It is through his guidance that I have developed as a scientist. I want to thank him for his unending support and assistance which has helped over come many obstacles encountered during my research. His unwavering enthusiastic support is greatly appreciated.

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I would like to thank Yuetong Wei for growing all of the different cell lines used in this research.

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I would like to thank my family and friends for their support as I pursued my Ph.D.

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INTRODUCTION

Overview

A major limitation in the treatment of cancer is the inability to diagnose a malignancy at an early, potentially curable stage of development. The tumorigenic process is complex, and the mechanisms involved in tumor development are not clearly understood. Research is needed to define differences between malignant and nonmalignant cells. The identification and characterization of specific tumor markers may be beneficial in developing novel diagnostic tests to detect malignancies (tumors) in an early developmental stage.

Tumor markers can be utilized as diagnostic and/or prognostic factors for malignancy. Diagnosis of malignancy may be based on the presence of a tumor marker and ideally this marker can predict the clinical course of the disease. To be used as a diagnostic tool, tumor markers must have high specificity. As prognostic indicators tumor markers can be used to predict recurrence of malignancy, disease free survival and overall survival. The therapeutic regimen and goals for a patient can be determined according to the presence or absence of certain markers. To be useful as a prognostic factor, tumor markers do not need to exhibit absolute specificity to be clinically relevant.

The selection of an ideal tumor marker should be based on certain criteria (Gao *et al.*, 1997; Keesee *et al.*, 1996; Schwartz *et al.*, 1993). The marker should be associated with a malignancy and should be easily detectable by analysis of body fluids or tissues. Healthy individuals and patients with benign conditions should not test positive for the tumor marker. Detection of a tumor marker also should facilitate the early detection of a malignancy, ideally before the tumor becomes clinically evident. The concentration of an ideal tumor marker in biological specimens should relate directly to tumor burden and correlate with tumor progression or regression in patients undergoing anticancer

therapy. Sensitivity and specificity are also important considerations in selecting tumor markers for the diagnosis of malignancies. Sensitivity refers to the percentage of cases in which a malignancy is correctly diagnosed and a tumor marker is present (Bates and Longo, 1987). Specificity refers to the ability of a tumor marker to exclude malignancy when not present (Bates and Longo, 1987). The measurement of sensitive and specific tumor markers is essential for reliable, qualitative identification of different malignancies.

The identification of all malignancies by a common tumor marker is not currently possible due to considerable heterogeneity of cells within individual tumors as well as between different tumors. The molecular and biochemical alterations involved in the complex oncogenic process can differ between tumors and between individuals diagnosed with the same malignancy, resulting in unique malignant phenotypes. This variety of molecular alterations contributes to the heterogeneity of malignancies.

Circulating Tumor Markers

Circulating tumor markers are defined as analytes in body fluids (i.e., blood and urine) that can be related to the presence of a malignancy (Pandha and Waxman, 1995). This definition has been expanded to include cytogenetic markers, oncogenes and abnormally expressed proteins. Bence-Jones discovered the first tumor marker in 1847. The investigator identified a precipitate in urine collected from patients diagnosed with "softening of the bones." This precipitate was later identified as the monoclonal light chain of immunoglobulin that is associated with multiple myeloma (Paredes and Mitchell, 1980; Kyle *et al.*, 1972). Numerous tumor associated antigens have been identified since the initial discovery of tumor markers.

Carcinoembryonic antigen (CEA) was first discovered in extracts of colonic adenocarcinomas by Gold and Freedman (1965). CEA is an oncofetal

antigen that is classified as a glycoprotein. Bates and Longo (1987) reported that patients with colon cancers had increased concentrations of CEA compared to healthy patients, and CEA levels were correlated with progression of the malignancy. Other investigators (Hayes, 1996; Schwartz *et al.*, 1993; Bates and Longo, 1987) reported that patients with breast, ovarian, and respiratory cancers also have elevated levels of CEA. However, Bates and Longo (1987) reported that CEA concentrations in these malignancies were not correlated with the progression of the malignancy and should not be used as a prognostic marker. Further research demonstrated that CEA concentrations vary considerably in individuals that smoke and in patients with benign liver and breast diseases (Hayes, 1996; Schwartz *et al.*, 1993; Bates and Longo, 1987; Pandha and Waxman, 1995). Van der Schouw *et al.* (1993) indicated that CEA is not a sensitive marker for early detection of colon cancer. The investigators reported elevated CEA levles in only 18-28% of patients with early stage colon cancer. CEA concentrations are currently monitored as a prognostic marker in colon cancer, but these tests fail to detect 40% of patients with late stage resectable tumors (Bates and Longo, 1987). Overall, CEA does not appear to be a reliable marker for diagnosis of colon cancer, but this marker may be beneficial in assessing patient prognosis and in determining treatment regiments.

Alphafetoprotein (AFP) is an oncofetal antigen that was first discovered in 1963 by Abelev. The presence of AFP in healthy adults is uncommon with the exception of pregnant women. AFP concentrations are elevated in patients with hepatocellular carcinomas, testicular teratocarcinoma and other embryonic malignancies compared to healthy individuals (Pandha and Waxman, 1995; Bates and Longo, 1987). Increased AFP concentrations have been found in some benign liver diseases and in some pancreatic, gastric, colonic, and lung malignancies (Bates and Longo, 1987). AFP levels alone are not sufficient to

provide prognostic information, but are useful in monitoring germ cell malignancies when combined with another tumor marker, human chorionic gonadotropin (hCG). hCG is a glycoprotein hormone normally produced only during pregnancy (Pandha and Waxman, 1995; Bates and Longo, 1987). hCG is an excellent tumor marker for trophoblastic tumors (Keesee *et al.*, 1996; Bates and Longo, 1987). The levels of hCG are raised in close to 100% of all trophoblastic tumors and hCG levels correlate with tumor burden (Keesee *et al.*, 1996; Pandha and Waxman, 1995; Bates and Longo, 1987). In addition, hCG levels also correlate with response to therapy and the presence of disease recurrence (Pandha and Waxman, 1995; Bates and Longo, 1987). When used together, hCG and AFP are excellent markers for detecting residual disease in nonseminomatous germ cell testicular cancer (Pandha and Waxman, 1995; Bates and Longo, 1987).

There are several members of a mucin like family of high molecular weight glycoprotiens that are secreted in malignant states. These include CA15-3, CA549, CAM26/29, and MSA. CA15-3 is elevated in some breast cancers, but this marker is not useful in the diagnosis of women with early stage or nonmetastatic breast cancer (Schwartz, 1994; Soletormos *et al.*, 1993). However elevated levels of CA15-3 were found in 63% of metastatic breast cancer patients and 71% of women with bone metastasis (Schwartz, 1994). As a prognostic marker CA15-3 levels are useful in monitoring patients for therapeutic response and recurrence of breast cancer (Pandha and Waxman, 1995; Schwartz, 1994; Soletormos *et al.*, 1993). Another marker, CA549, was found to have a high specificity (98%) but a relatively low sensitivity in advanced breast cancer (Schwartz, 1994). The mucin type markers are not specific for breast cancer since elevated levels are found in benign breast and liver conditions as well as other malignancies (Hayes, 1996). In general the mucin type antigens

are not effective in detecting early stage breast cancer but may be used to monitor for recurrence of disease (Pandha and Waxman, 1995; Schwartz 1994; Soletormos *et al.*, 1993; Magdelenat, 1992).

Prostate specific antigen (PSA) is a glycoprotein produced by the ductal epithelial cells of the prostate gland. PSA has replaced prostate acid phosphatase (PAP) as a diagnostic and prognostic marker for prostate cancer due to its high sensitivity (96%) and specificity (97%) (Pandha and Waxman, 1995). The FDA has recently approved the use of PSA for the diagnosis of early stage prostate cancer. Levels of PSA correlate with both tumor burden and androgen levels and can be used to monitor response to treatment (Magdelenat, 1992). PSA levels are also useful in detecting residual disease after surgery and recurrence of the malignancy (Pandha and Waxman, 1995; Magdelenat, 1992). PSA is one of the best tumor markers available due to its high sensitivity and specificity as well as its correlation with the presence of malignancy. However a main drawback is its inability to distinguish between prostate cancer and benign prostate conditions (Pandha and Waxman, 1995; Magdelenat, 1992). PSA levels also are not reliable in the diagnosis and treatment of hormone refractory prostate cancer (Pandha and Waxman, 1995; Magdelenat, 1992).

CA125 is a large carbohydrate antigen used as a tumor marker for ovarian cancer (Schwartz, 1993; Magdelenat, 1992). CA125 has a high tumor sensitivity (67%) but a low specificity (Woolas *et al.*, 1993). The marker is not useful in detecting early stage ovarian cancer but levels of CA125 correlate with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). Increases in CA125 levels can be used to monitor for recurrence of disease (Pandha and Waxman, 1995; Schwartz, 1993). Levels of CA125 are elevated in other malignancies as well as in benign breast, liver, and ovarian conditions (Schwartz, 1993).

There are several other markers currently being used in the diagnosis and treatment of malignancies. Calcitonin is approved by the FDA for the screening and monitoring of medullary thyroid carcinoma (Bates and Longo, 1987). Immunoglobulin, the precipitate first identified by Bence-Jones, is used in the diagnosis of multiple myeloma. The initial levels of immunoglobulin are used to predict survival and response to therapy (Bates and Longo, 1987). Elevated levels of catecholamine metabolites, vanillylmandelic acid and homovanillic acid, are used in the diagnosis of neuroblastoma (Bates and Longo, 1987). Nuclear mitotic apparatus protein (NuMA) has recently been approved by the FDA for the diagnosis of bladder cancer.

The circulating tumor markers described above have several limitations. CEA, AFP, CA125 and the mucin like markers are poor diagnostic markers because they are not detected in early stage malignancies. These markers are also found to be elevated in other malignancies as well as in benign conditions. These limitations prevent these markers from being used as diagnostic screens for cancer (with the exceptions of PSA, hCG, calcitonin and NuMA), but, on an individual basis, they may be useful as prognostic markers.

Molecular Tumor Markers

In recent years there has been much research focused on molecular tumor markers. These markers include tumor suppressor genes and oncogenes. These genes have a critical role in the regulation of cell proliferation. Mutations in these genes may result in dysregulation of cell division, which may lead to malignancy. Molecular markers are being used to screen populations to identify individuals with an increased risk of developing a malignancy, to predict patient prognosis and in the determination of the most appropriate therapeutic regiment for individual patients.

One of the most extensively studied molecular tumor markers is the tumor suppressor gene p53. Mutations in the p53 gene are found in 50%-80% of all cancers (Ruteshouser and Hansen, 1995). The use of p53 as a tumor marker is complicated by the number of different mutations which may affect the gene. The protein product of the p53 gene has multiple and poorly understood functions (Schwartz *et al.*, 1993). In breast cancer p53 overexpression has been shown to correlate with negative estrogen receptor status and high nuclear grade, which are indicators of poor patient prognosis (Schwartz *et al.*, 1993). Overexpression of p53 is found in some prostate cancers. This characteristic is more common in advanced stage prostate cancer, but its presence correlates with disease recurrence after surgical removal of the primary tumor (Abbus and Scardino, 1997; Yang *et al.*, 1996; Moul *et al.*, 1996;). p53 is also associated with an increase in cell proliferation, stage, grade and androgen independence in prostate cancer (Lahani *et al.*, 1994).

The ras oncogene is another molecular marker that has received much attention. The ras gene product is a small GTPase that is involved in growth promoting signal transduction (Deyo *et al.*, 1995). The incidence of ras mutations varies widely between different types of cancer as well as between cancers of similar origin (Deyo *et al.*, 1995). The highest incidence of ras mutations are found in pancreatic malignancies, where the mutations occur in 75% - 90% of tumors (Deyo *et al.*, 1995). In both lung and colon cancers ras mutations are associated with poor prognosis. Although ras mutations are present in prostate tumors, there is no correlation between the mutations and the initiation or progression of malignancy (Peehl, 1993). The Her2/neu gene is also found to be overexpressed in cancer. Her2/neu has been most extensively studied in breast cancer. Overexpression of the gene is found in 30% of breast tumors and is associated with poor clinical outcome (Schwartz *et al.*, 1993).

Mutations in the BRCA 1 tumor suppressor gene have been associated with as increased risk of breast and ovarian cancer. BRCA 1 mutations are commonly found in families with a history of breast and/or ovarian cancer (Johannsson *et al.*, 1998; Vehmanen *et al.*, 1997). There have been more than 300 reported mutations to the BRCA 1 gene, most of which result in a truncated protein product (Garcia-Patino *et al.*, 1998; Petriji-Bosch *et al.*, 1997; Xu and Solomon, 1996). The protein product of BRCA 1 is a nuclear protein which acts as a transcription factor (Johannsson *et al.*, 1998). The protein interacts with p21 contributing to cell cycle arrest (Johannsson *et al.*, 1998). The survival of cancer patients who are carriers of the mutated BRCA 1 is similar to or worse than the survival of breast and ovarian cancer patients in general (Johannsson *et al.*, 1998b). The BRCA 1 gene is useful for the identification of individuals who are at high risk for breast and ovarian cancer, and should be monitored carefully for the development of malignancy.

These are just a few examples of molecular markers currently being investigated for their role in the tumorigenic process. With the exception of BRCA 1 these markers are not used in the diagnosis or screening of malignancy due to their low specificity and sensitivity. As a group the molecular tumor markers are useful prognostic factors for specific malignancies. However, further research is needed to evaluate the role of these genes in the development of malignancy.

Histological Analysis of Tumors

The diagnosis of a malignancy is made after microscopic examination of biopsy material or resected tissue. Tissue is routinely evaluated for a number of markers including the number of mitotic figures present, tissue architecture, cellularity and nuclear pleomorphism (Rosai, 1993). The information derived from the microscopic examination of the biopsy material is used by clinicians to

determine histologic stage and grade of the tumor, and this information along with the lymph node status is useful for determining patient prognosis and therapeutic response. Tumor size, histological grade and nodal status are considered the most important prognostic factors in newly diagnosed breast cancer (Hayes, 1996; Haerslev *et al.*, 1996; Schwartz *et al.*, 1993). These parameters are also used to evaluate the risk of tumor recurrence (Schwartz *et al.*, 1993). Similarly, in prostate and colon carcinomas, histopathological methods are the best prognostic indicators currently available to the clinician.

In addition to gross microscopic examination there are other factors which are used as prognostic markers. DNA content, S phase fraction, and the proliferation rate are used as prognostic indicators in many kinds of malignancy (Meyers, 1991). DNA aneuploidy is found in many colon adenocarcinomas and has been correlated with a decrease in survival (Schutte *et al.*, 1982). In breast cancer high S phase content and DNA aneuploidy correlate with poor patient prognosis (Schwartz *et al.*, 1993; Kallioniemi *et al.*, 1988 ; 1987). It has been demonstrated that the proliferative index of colon tumors is increased when this parameter is compared to that of the normal colon mucosa (Zusman, 1995).

The rate of proliferation of a tumor can be determined by S phase content, thymidine labeling, and antibody staining with KI-67 or PCNA. Proliferating cell nuclear antigen (PCNA) is a nuclear protein that is an accessory factor for polymerase δ during DNA synthesis and is therefore a good measure of cell proliferation. The levels of PCNA are determined by histological staining of tissue slices. PCNA is strongly expressed in malignant breast and prostate cells (Meyers and Grizzle, 1997; Haerslev *et al.*, 1996; Schwartz *et al.*, 1993; Tuccari *et al.*, 1993). Perry and Tindall (1996) reported that androgens play an important role in prostate cell proliferation through the posttranscriptional regulation of PCNA. There is also evidence that PCNA expression may be an early event in

the tumorigenic process. Rustgi (1997) reported that premalignant esophageal cells demonstrate strong PCNA staining compared to nonmalignant cells. There is also evidence that PCNA is present in premalignant prostate lesions (Meyers and Grizzle, 1997).

Tumor markers represent potential diagnostic indicators of malignancies. Monitoring tumor markers in cancer patients may serve as a beneficial prognostic technique to assist clinicians in predicting the potential for recurrence of a malignancy. The treatment regiment for a cancer patient may also be determined by assessing whether certain tumor markers are present. Currently, only six tests for tumor markers have been approved by the US Food and Drug Administration for the diagnosis of specific malignancies (Table 1). Many of the tumor markers in Table 1 do not possess adequate specificity and sensitivity to detect malignancies and are therefore not useful as a screening tool for the early detection of malignancies.

This research project was designed to identify proteins which differ between malignant and nonmalignant cells. The project has focused on the proteins required for DNA synthesis because of their critical role in cellular proliferation. This report will discuss the identification and characterization of a cancer specific form of PCNA.

Table 1

Clinical Applications of Useful Tumor Markers

Cancer	Markers [a]	Recommended Application [b]
Breast	CEA [c], CA 15.3, CA549, MCA etc.	M
Colon	CEA [c]	P,M
Pancreatic, gastric	CA 19.9, CA 72.4, CA 50	M
Hepatocellular	AFP [c] CEA [c]	S,D,P,M M
Prostate	PSA [c] PAP [c]	D,P,M P,M
Ovary	CA125 [c]	P,M
Testicular	AFP [c], hCG LDH [c]	D,P,M P,M
Trophoblastic	hCG	D,P,M
Thyroid	Thyroglobulin [c] Calcitonin [c] (Medullary)	S,M, D,M
Neuroblastoma	NSE Catecholamines [c]	M S,D,P,M
Multiple Myeloma	Immunoglobulins [c]	D,P

[a] Represents a partial listing of circulating tumor markers in worldwide use.

[b] S, screening; D, diagnosis; P, prognosis; M, monitoring course of disease or response to therapy.

[c] FDA approved.

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Chapter II

TITLE:

A UNIQUE FORM OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IS PRESENT IN MALIGNANT BREAST CELLS

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Running Title:

PCNA in Breast Cancer

Key Words:

Breast Cancer, PCNA, DNA Replication, DNA synthesome, Post Translational Modification, Diagnostic, Biomarker, Tumor Marker

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Abstract

Despite extensive research efforts to identify unique molecular alterations in breast cancer, to date no characteristic has emerged that correlates exclusively with malignancy. Recently it has been shown that the multiprotein DNA replication complex (synthesome) from breast cancer cells has a significantly decreased replication fidelity compared to that of nonmalignant breast cells. Proliferating cell nuclear antigen (PCNA) functions in DNA replication and DNA repair, and is a component of the synthesome. Using 2D PAGE analysis a novel form of PCNA has been identified in malignant breast cells. This unique form is not the result of a genetic alteration, as demonstrated by DNA sequence analysis of the PCNA gene from malignant and nonmalignant breast cells. This novel form is most likely the result of an alteration in the post-translational modification of PCNA in malignant breast cells. These findings are significant in that it is now possible to link changes in the fidelity of DNA replication with a specific alteration of a component of the DNA synthetic apparatus of breast cancer cells. The novel form of PCNA may prove to be a new signature for malignant breast cells.

Introduction

Breast cancer is a leading cause of death among women. In 1996 there were approximately 44,000 deaths attributed to breast cancer[1]. Breast tumors show a strong correlation between high rates of DNA synthesis and poor over-all patient prognosis. Data suggest that high levels of breast cancer cell DNA synthesis are associated with an increased probability of lymph node metastases[2-7]. Extensive genetic damage in mammary cancer cells is also associated with increased levels of DNA synthesis. An evaluation of thousands of clinical cases indicated that approximately 65% of mammary cancer cells are aneuploid[8]. Therefore, the observed high levels of DNA synthesis and extensive genetic damage in breast tumors strongly suggests that an alteration in the DNA replication machinery of these cells may contribute to uncontrolled and error-prone DNA synthesis.

We have recently found that human breast cells mediate DNA synthesis using a multiprotein replication complex, designated the DNA synthesome[9]. The human breast cell DNA synthesome is fully competent to support SV40 *in vitro* DNA replication. Recently it was found that the fidelity of DNA synthesis mediated by the synthesome isolated from the malignant breast cells is several-fold lower than the synthesome isolated from the nonmalignant breast cells[9-10]. The decreased replication fidelity of the malignant breast cell DNA synthesome clearly suggests a critical alteration(s) in the composition of the replication machinery of these cells.

Proliferating cell nuclear antigen (PCNA) is associated with the human cell DNA synthesome[11] and is required for synthesome mediated *in vitro* DNA replication[12]. PCNA has been demonstrated to function in mammalian cell DNA replication (reviewed in 13) and DNA repair[14-15]. In DNA replication PCNA serves as an accessory factor to polymerase δ. PCNA is also commonly used as a

proliferation marker in breast cancers. Unfortunately, there has been no conclusive evidence that increased levels of PCNA correlate with tumor progression or patient prognosis[16-18]. In this study the DNA synthesome associated PCNA, isolated from nonmalignant and malignant breast epithelial cells, was analyzed by two dimensional gel electrophoresis (2D PAGE). In this report, we describe for the first time that a unique form of PCNA is found in all malignant breast cells. This is an exciting finding which may prove to be a new signature for malignant breast cells.

Results

A Unique Form of PCNA Identified in Malignant Breast Cell Cultures

Studies were performed to determine whether DNA synthesome components are structurally altered in malignant breast cells compared to nonmalignant cells. The DNA synthesome was isolated from four established breast cells lines (MCF 7, MDA MB 468, Hs578T and early passage MCF 10A) as well as from nonmalignant primary breast epithelial cells using our published procedures[9]. The malignant breast cells lines (MCF 7, MDA MB 468 and Hs578T) produce tumors in animal breast cancer models[19], while the nonmalignant breast cell line, MCF 10A, does not[20]. The nonmalignant primary breast cells were prepared from a human breast reduction sample as described by Stampfer[21]. Thirty micrograms of DNA synthesome isolated from MCF 10A, MCF 7, MDA MB 468, Hs578T and nonmalignant primary breast cells were each subjected to individual 2D PAGE[22-23]. These gels, containing the resolved synthesome polypeptides, were transferred to nitrocellulose membranes. Western blot analyses of the membranes were performed using an antibody directed against the 36 kDa PCNA polypeptide. A comparison of the mobility of the PCNA component of the MCF 10A, MCF 7, MDA MB 468, Hs578T and

primary cell derived DNA synthesome (Figure 1) indicated a clear and significant difference in this protein's 2D PAGE profile for the nonmalignant and malignant cells. The PCNA associated with the synthesome isolated from malignant MCF 7 and MDA MB 468 cells was present in two forms, a basic form and an acidic form (Figure 1 A, E). The PCNA isolated from the malignant Hs578T cells exhibited PCNA with an acidic pI (Figure 1C) and barely detectable levels of PCNA with a basic pI. PCNA in nonmalignant MCF 10A and primary breast cells was present in a single form that exhibited a basic pI (Figure 1B,D). The PCNA form with the acidic pI was not detectable in the nonmalignant cells.

In a separate experiment 2D PAGE analysis was performed using a sample containing isolated DNA synthesome from both MCF 7 and MCF 10A cells. The resulting protein migration pattern showed only one basic form and one acidic form of PCNA (data not shown). This result indicated that the basic form of PCNA was identical in both the malignant and nonmalignant cells; while the acidic form of PCNA was unique to the malignant cells.

Breast Tumors Contain the Unique Form of PCNA

Studies were initiated to determine whether the DNA synthesome derived from nonmalignant and malignant breast tissue exhibited the same 2D PAGE profile for PCNA as was observed in the nonmalignant and malignant breast cell cultures. The DNA synthesome was isolated from a virally induced mouse breast tumor[24]. The DNA synthesome isolated from six human lobular breast cancer tissues and from four ductal breast cancer tissues was also analyzed. For comparison, the DNA synthesome associated PCNA isolated from nonmalignant breast tissue was examined from two sources (breast reduction tissue and genetically matched nonmalignant tissue taken from the patients with malignant breast tumors). The purified DNA synthesome derived from these tissues was

resolved by 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and probed with an antibody directed against PCNA. PCNA derived from malignant mouse and human tumor tissue had a 2D PAGE profile consistent with that of the malignant breast cell lines (Figure 2 A-D,F). There were two forms of PCNA present, an acidic form and a basic form. The PCNA from the nonmalignant breast tissue was in the basic form (Figure 2E). These findings were consistent with the 2D PAGE profile of PCNA from the MCF 10A cells.

The Unique Form of PCNA is not Proliferation Dependent

To demonstrate that the abundant levels of the form of PCNA with a acidic pI was a property unique to malignant breast cells as opposed to a proliferation response, we analyzed the 2D PAGE profile of PCNA isolated from benign proliferative breast tumors and estrogen stimulated MCF 7 cells. Estrogen has a stimulatory effect on cellular proliferation[25]. We found that the estrogen stimulated cells had an increased rate of proliferation compared to control cells as demonstrated by several parameters (Table 1). Similar findings have been reported by other investigators[25-27]. The DNA synthesome was isolated from these cells, and the components resolved by 2D PAGE followed by Western analyses for PCNA. There was an overall increase in the level of PCNA in estrogen stimulated cells, however, there was no effect on the 2D PAGE profile for PCNA (Figure 3A,B).

The DNA synthesome was also isolated from several benign breast tumors. In general, the 2D PAGE profile for PCNA from the benign tumors was identical to that of nonmalignant cells in culture and nonmalignant breast tissue (Figure 3C). These data provide compelling evidence that the acidic form of PCNA is characteristic of malignant breast cells.

Genetic Mutation Is not Responsible for the Acidic Form of PCNA in Malignant Breast Cells

Total cellular RNA isolated from MCF 7 and MCF 10A cells was used to clone the cDNA encoding the entire PCNA translation unit from each cell line. Nucleotide sequence analysis of four independent clones encoding the PCNA gene derived from MCF 7 cells and four independent clones from MCF 10A cells were sequenced. Sequence analysis indicated that these eight independent clones have an identical nucleotide sequence (Figure 4). Furthermore, this nucleotide sequence does not differ from that of the sequence for the PCNA gene cloned from the human lymphoma cell line MOLT-4[28] (Figure 4).

Discussion

In this report it was demonstrated that a unique form of PCNA with an acidic pI is present in malignant breast cells. This unique form is found in malignant breast cell cultures as well as malignant breast tumors but not in nonmalignant breast cell cultures, nonmalignant tissue or a sampling of benign breast tumors. It was shown that the cancer specific form of PCNA was not due to a proliferation response or genetic mutation. This novel form of PCNA is most likely a result of an altered post-translational modification in the malignant breast cells.

One of the hallmarks of breast cancer is the accumulation of genetic mutations which lead to genomic instability[29]. These mutations may contribute to uncontrolled cellular proliferation, resistance to antiproliferative processes and metastasis[4,7,30]. The accumulation of mutations in breast cancers is high considering that normally mutations occur infrequently. This has lead to the hypothesis of a "mutator phenotype"[31]. It was proposed that a cell's progression

to malignancy is accompanied by the accumulation of multiple genetic mutations created by error-prone DNA replication and a reduction in the efficiency of the DNA repair processes in the cell. Sekowski *et al.* (submitted)have recently found that the DNA replication apparatus from malignant breast cells is mutagenic, resulting in a decreased replication fidelity. Due to the essential role PCNA plays in both DNA replication and DNA repair, the unique form of PCNA in malignant cells is a likely contributor to the accumulation of genetic mutations and genomic instability.

An implication of the described findings is that post-translation modification of PCNA is most likely responsible for the development of the acidic form of the protein found in the cancer cells. Bravo and Celis (1985) demonstrated that PCNA from HeLa cell extracts was not post-translationally modified by phosphorylation and that acetylation of glycosylation and sialylation are not likely contributors to the migration pattern of PCNA. Simbulan *et al.* (1996) have recently shown that PCNA is modified by poly(ADP) ribosylation. Further studies to determine how the post-translational modification of PCNA affects its ability to interact with proteins involved in DNA replication and DNA repair are underway and should provide crucial insights into the role of PCNA in the maintenance of genomic stability and control of cellular proliferation.

Another implication of this work is that the malignant cell specific form of PCNA may be a more fundamental characteristic of cancer cells than any single type of genetic mutation. This supposition is based on the observation that the cancer specific form of PCNA was found in 100% of malignant breast cells examined to date. This correlation is higher than that observed for other molecular markers for malignancy currently examined for in breast tumors (i.e., p53, BRCA, her2/neu, progesterone and estrogen receptor status, which are only found in a fraction of all breast tumors.

The findings described in this report are novel in that this is the first time it has become possible to link the decreased fidelity of cellular DNA replication with a specific alteration to a component of the DNA synthetic apparatus of breast cancer cells. PCNA is currently used as a marker for cellular proliferation. The novel form of PCNA, which we have identified, may prove to be a new signature for malignant breast cells. Our findings provide the first evidence that the unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of malignancy.

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Methods

Cell culture

MCF 7, early passage MCF 10A, MBA MD 468, and Hs578T cells were cultured according to protocols provided by American Type Culture Collection (ATCC). Primary cells were cultured in Mammary Epithelial Growth Medium (Clonetics, San Diego, CA) supplemented with 2X penicillin/ streptomycin, 2.5 mg/ml amphotericin B, 50 U/ml polymixin B sulfate, 50 mg/ml gentamicin sulfate, 10 ng/ml EGF, 5 mg/ml insulin, 0.5 mg/ml hydrocortisone, and 52 mg/ml bovine pituitary extract and grown as described previously[21]. To stimulate MCF 7 cells with estrogen, cells were grown in media containing charcoal treated serum. Cells were treated for 48 hour with 1 nM 17 β estradiol or an equivalent volume of vehicle. Cells were harvested and the DNA synthesome isolated as described previously [9].

Isolation and purification of the DNA synthesome from breast tumor tissue and breast cell lines

The mammary mouse tumor was induced using polyoma virus as described previously[24]. The isolation and purification of the DNA synthesome were performed as described previously [9].

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE)

DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient created using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide

SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis

The antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Immunoprecipitation of PCNA

One hundred micrograms of isolated DNA synthesome were incubated overnight with a PC10 antibody directed against PCNA. Thirty µl of protein A conjugated agarose beads were added to the reactions for 1 hr. The reaction mixtures were washed twice with buffer A (0.154 M NaCl, 10 mM Tris-HCl pH 7.4, 0.05% Triton X 100, 0.05% SDS) and three times with buffer B (0.154 M NaCl, 50 mM Tris-HCl pH 7.4, 2.5M KCl, 0.5% Triton X 100). The protein was removed from the beads by incubation at 100°C for 30 sec and analyzed by 2D PAGE.

Mutational analysis of the PCNA gene

The cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand cDNA Synthesis (Gibco/BRL) followed by cloning of the amplified cDNA into the vector pCR2.1 (*In vitro*gen) according to the manufacturer's instructions. Total RNA was isolated using Trizol reagent (Gibco/BRL). Second strand cDNA synthesis was carried out by priming the first strand cDNA with oligonucleotide 5'GCGTTGTTGCCACTCCGC3' on the 5' end of the cDNA, and 5'GCAGTTCTCAAAGAG-CTTAG3' on the 3' end of the cDNA, and amplifying the primed first strand using RT PCR.

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Figure Legends

Figure 1. Protein migration of PCNA from human breast cell lines. Thirty micrograms of DNA synthesome protein isolated from four human breast cell lines (MCF 7, MDA MB 468, Hs578T, and MCF 10A) and nonmalignant primary breast cells were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis with an antibody directed against PCNA. The protein migration patterns shown are: (A)MCF 7, (B) MCF 10A, (C) Hs578T, (D) nonmalignant primary breast cells and (E) MDA MB 468. The arrow indicates the form of PCNA unique to malignant breast cells.

Figure 2. Protein migration of PCNA from malignant human and mouse breast tissue and nonmalignant human breast tissue. Thirty micrograms of isolated DNA synthesome from malignant human and mouse breast tumors and nonmalignant human breast tissue were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis using an antibody directed against PCNA. The resulting protein migration patterns are shown: (A & B) human ductal tumor, (C & D) human lobular tumor (E) nonmalignant human breast tissue, and (F) mouse tumor. The nonmalignant breast tissue (E) is derived from the same source as the human lobular tumor in (D). The arrow indicates the form of PCNA unique to malignant breast cells.

Figure 3. Protein migration of PCNA from estrogen treated MCF 7 cells, control MCF 7 cells, and a benign breast tumor. Thirty to 60 micrograms of DNA synthesome isolated from MCF 7 cells treated with 17 β-estradiol, (A) MCF

7, (B) control cells, and (C) a benign breast tumor were analyzed by 2D PAGE and Western blot analysis. The arrow indicates the form of PCNA unique to malignant breast cells.

Figure 4. Nucleotide sequence of the PCNA gene cloned from MCF 7 and MCF 10A cells. The nucleotide sequence of the PCNA gene cloned from the two breast cell lines is aligned with the sequence reported for an acute lymphoblastic leukemia cell. The PCNA nucleotide sequences shown are those of: (A) MOLT-4, (B) MCF 7, and (C) MCF 10A. The positions of the ATG start codon and the internal BamHI restriction endonuclease cleavage site are underlined. The cDNA was cloned from total cellular RNA isolated from exponentially growing MCF 7 and MCF 10A cells using RT PCR and the pCR2.1 vector. Ampicillin-resistant colonies containing the cDNA were chosen using the blue/white assay, and miniprep DNA was isolated from the selected colonies and given to our Biopolymer Core Facility for nucleotide sequence analysis. The nucleotide sequences of the analyzed clones (4 from MCF 7 and 4 from MCF 10A) were identical to the sequence depicted in the figure.

TABLE 1. Stimulation of cell proliferation following treatment with 17 β-estradiol.

	Control Cells[a]	17 β-estradiol (E_2) Treated Cells[b]
^{3}H Thymidine Uptake[c]	1,548 cpm/10^5 cells	10,564 cpm/10^5 cells
DNA polymerase α Activity[d]	496 +/- 80 cpm/mg	1,359 +/- 118 cpm/mg
Cells in S Phase[e]	10.7%	60.1%

a) Control cells are MCF-7 that were grown in phenol red free DMEM, which was supplemented with 10% dextran coated, charcoal treated, fetal bovine serum, and 1% penicillin/streptomycin non-essential amino acids.

b) 17 β-estradiol (E_2) treated cells were grown for 48 hours under essentially the same conditions as the control cells along with the addition of 1 mM 17 β-estradiol to the media.

c) ^{3}H-thymidine uptake, according to the procedure described by Malkas *et al.* (1990).

d) DNA polymerase α activity was measured as described by Malkas *et al.* (1990).

e) Cell cycle distribution analyses of the cultured cells grown in the presence or absence of 17 β-estradiol were performed as described by Lin *et al.* (1997).

Figure 1

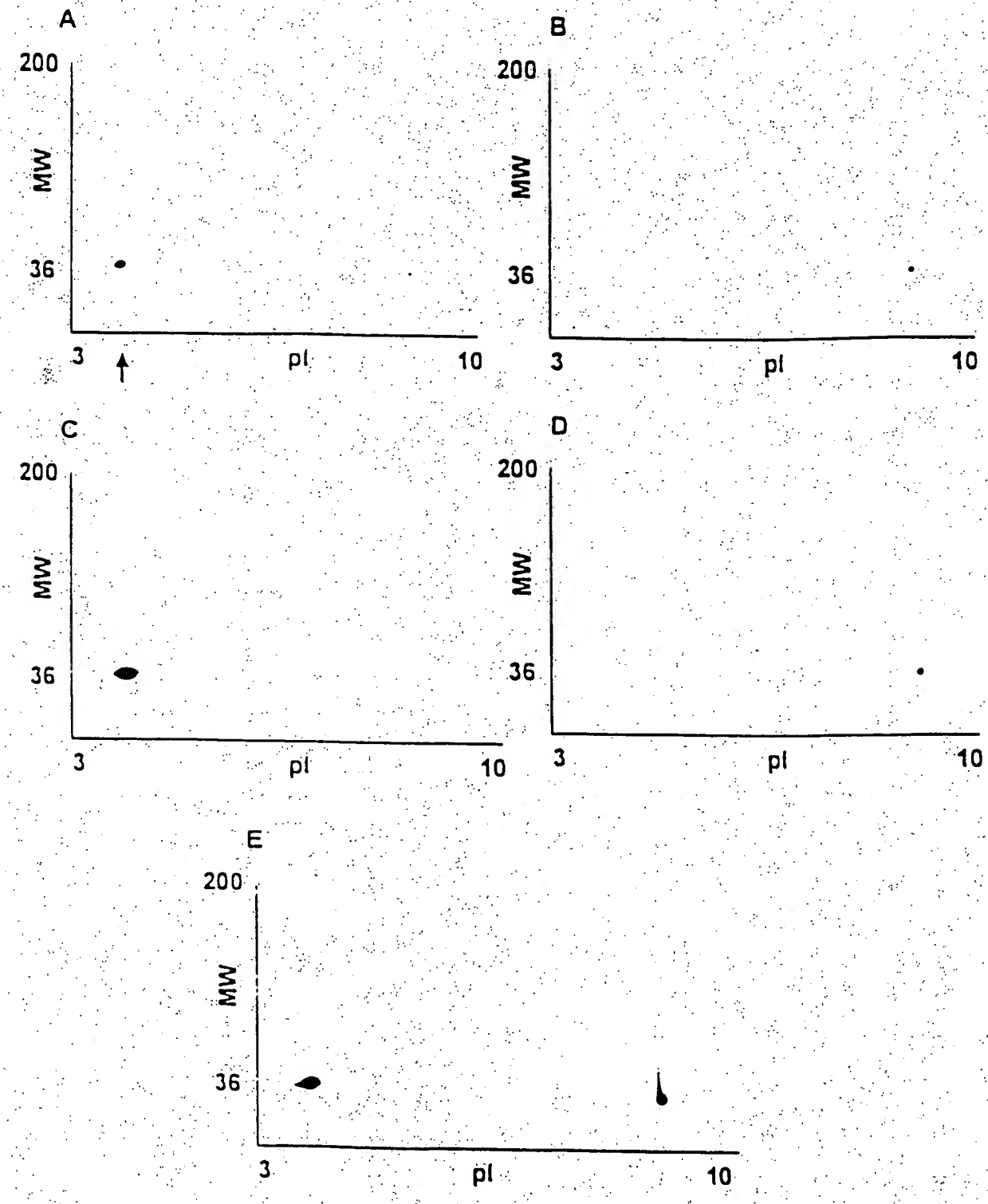
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Figure 2

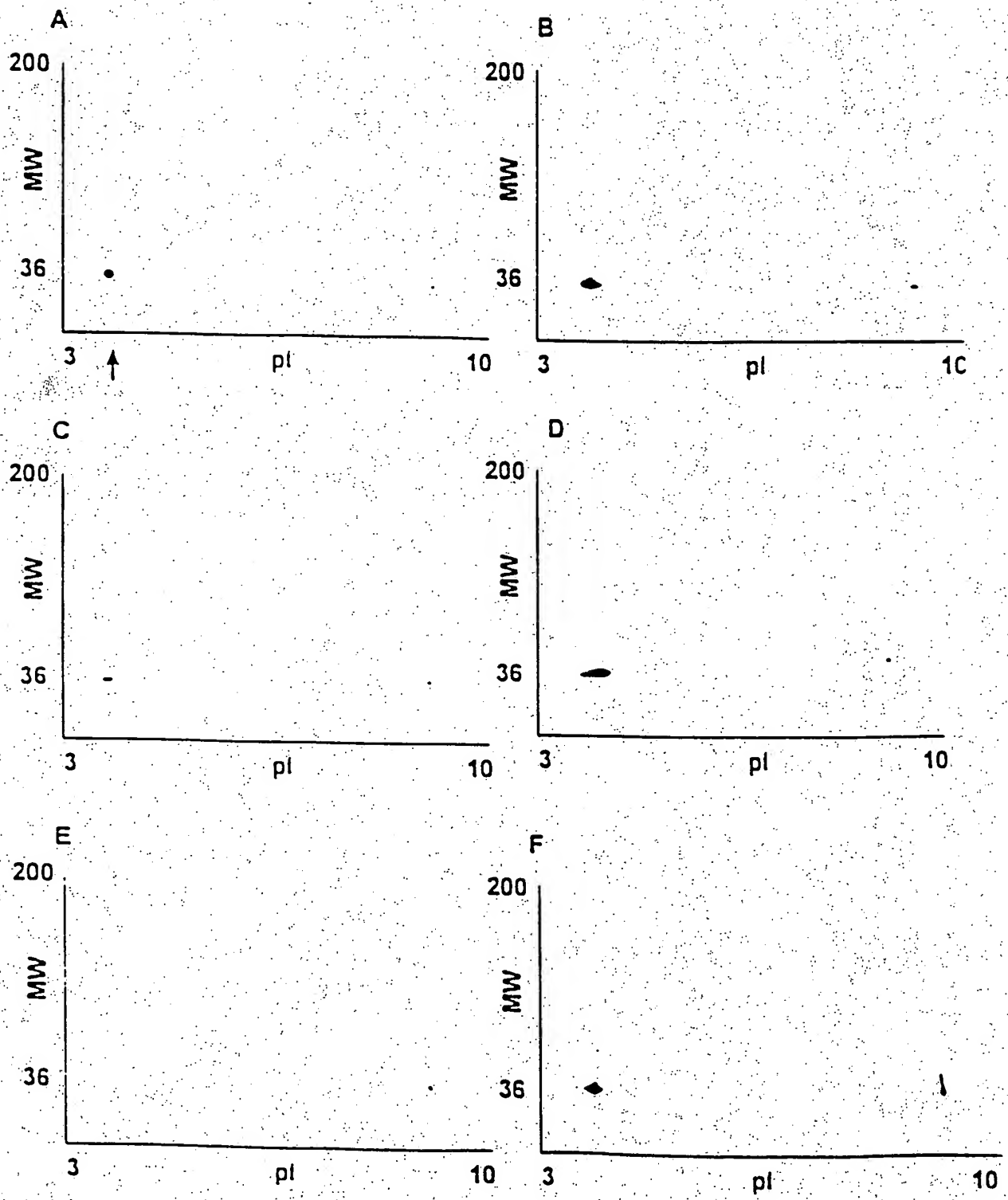
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Figure 3

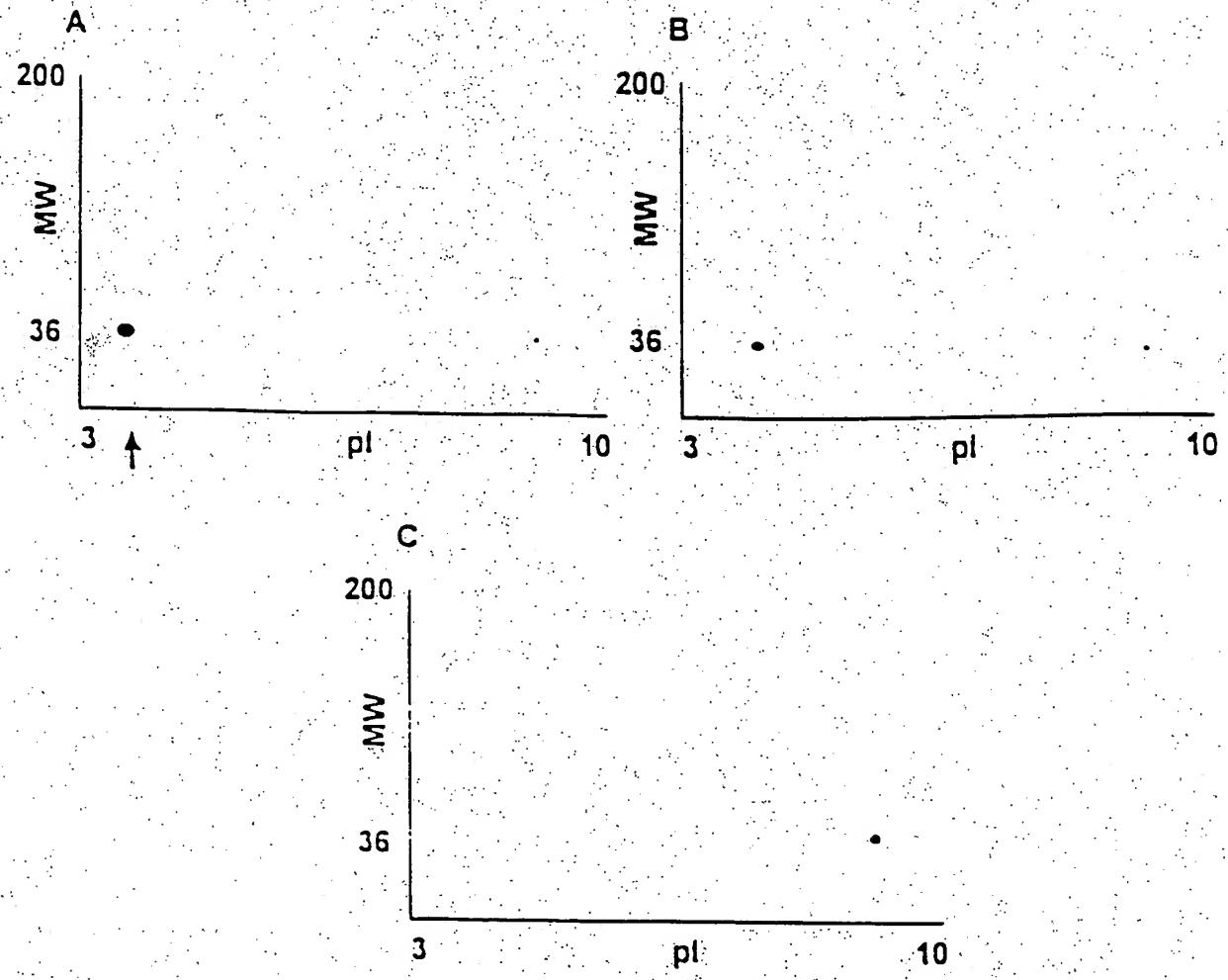
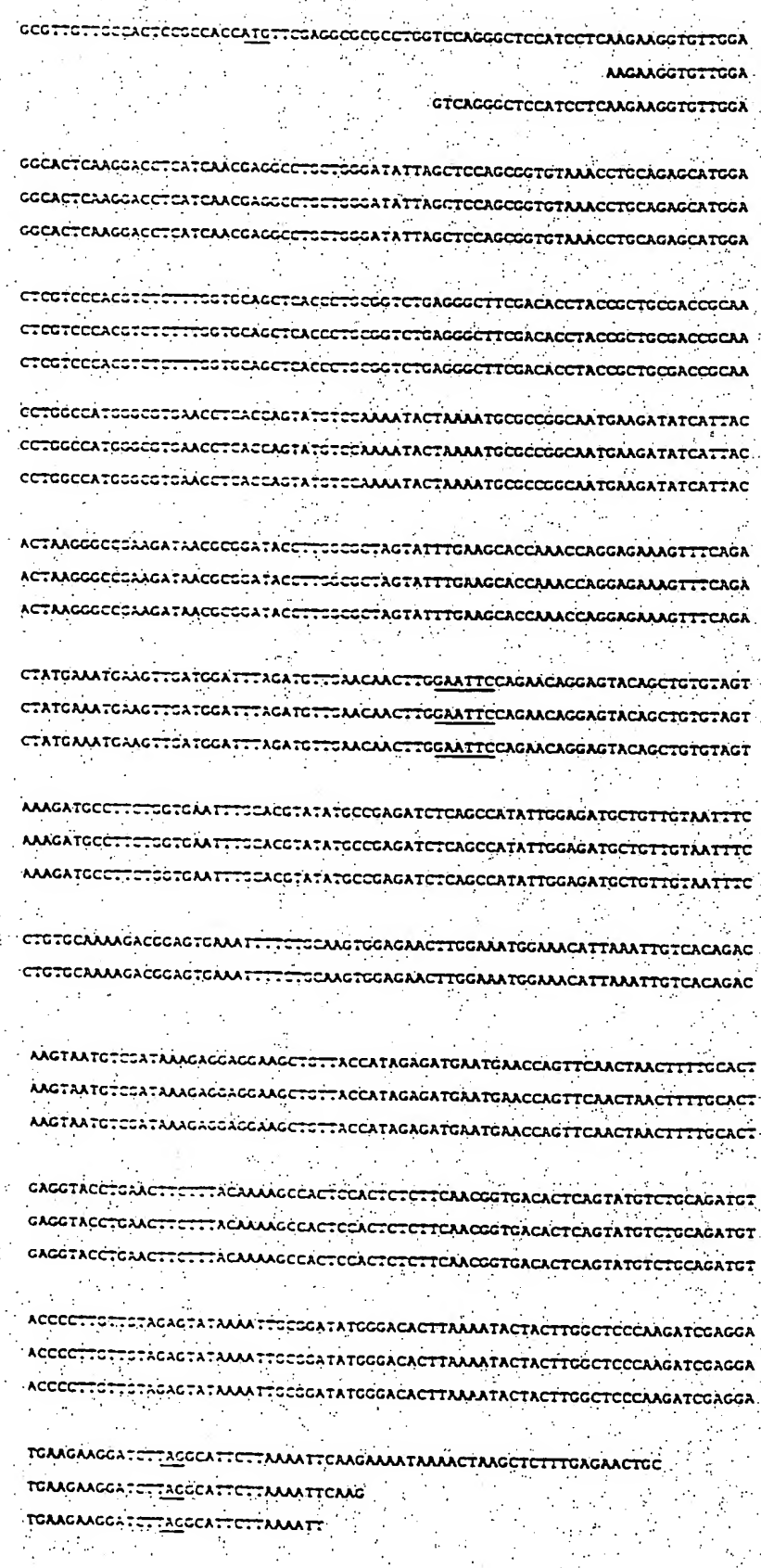


Figure 4



Chapter III

Chapter III

Title:

Characterization of an Acidic Form of Proliferating Cell Nuclear Antigen

Authors:

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Key Words:

PCNA, Poly(ADP-ribosylation), Tumor marker, Post-translational modification

Abstract

There has been considerable research directed at identifying characteristics which exclusively correlate with malignant breast cells. Recently, it was found that malignant breast cells contain a unique form of the protein, proliferating cell nuclear antigen (PCNA) which can be clearly distinguished from the form present in nonmalignant breast cells. PCNA is a 36 kD nuclear protein which is required for DNA replication and DNA repair. The unique form of PCNA is an acidic protein which results from epigenetic changes and is not a consequence of growth stimulation or genetic mutation. The purpose of this study was to further characterize this protein in transformed, but nontumorigenic breast cells, and to evaluate the molecular basis for the difference(s) between the basic form of PCNA and the cancer specific acidic form of the protein. PCNA isolated from the transformed, nontumorigenic cell lines 184A1N4myc (A1N4myc) and 184A1N4T (A1N4T) was analyzed by two dimensional gel electrophoresis (2D PAGE) for the presence of the unique form of PCNA. Serum samples collected from a breast cancer patient and cancer free individuals were also analyzed to determine whether the serum contained detectable levels of the cancer specific form of PCNA. PCNA was also isolated from MCF 7 cells in order to examine whether the basic and acidic forms of PCNA resulted from differential post-translational modifications of the polypeptide. The acidic form of PCNA was identified in the A1N4myc and A1N4T cell lines as well as in the breast cancer serum sample. The basic form of PCNA was determined to be poly (ADP-ribosylated), while the cancer specific acidic form was unmodified. These findings suggest that the charge difference between the basic and acidic forms of PCNA may be due to differential poly(ADP-ribosylation). These results also suggest that the altered form of PCNA may result from cellular

transformation events caused by the overexpression of c-myc and SV40 large T antigen.

INTRODUCTION

In breast tumors a strong correlation exists between high rates of DNA synthesis and poor over-all patient prognosis. High levels of breast cancer cell DNA synthesis are also associated with an increased probability of lymph-node metastases and extensive genetic damage (Meyer *et al.*, 1986; Kallioniemi et al, 1987; 1988; Hedely *et al.*, 1987; Klintenberg *et al.*, 1987; Sato *et al.*, 1991). Both the observed high rates of DNA synthesis and extensive genetic damage in breast tumors strongly suggest that an alteration in the DNA replication machinery of these cells results in uncontrolled and error-prone DNA synthesis.

A multiprotein replication complex, the DNA synthesome, has been isolated from human cells and tissues (Malkas *et al.*, 1990; Applegren *et al.*, 1995; Coll *et al.*, 1996). The DNA synthesome is composed of all of the proteins necessary for DNA replication (Figure 1) and is fully competent to mediate *in vitro* replication of SV40 origin containing DNA. Proliferating cell nuclear antigen (PCNA) is one of the protein components of the DNA synthesome, and is required for mammalian cell DNA replication as well as DNA repair (Hickey and Malkas, 1997; Coll *et al.*, 1996; Umar *et al.*, 1996; Applegren *et al.*, 1995; Malkas *et al.*, 1990).

PCNA is commonly used as a proliferation marker in the evaluation of breast cancer (Haerslev *et al.*, 1996; Schwartz *et al.*, 1993; Tuccari *et al.*, 1993, Kallioniemi *et al.*, 1988;1989). However, there has been no conclusive evidence that increased levels of PCNA correlate with tumor progression or patient outcome (Haerslev *et al.*, 1996; Schmitt et al, 1994). Recently, Bechtel *et al.* (submitted) performed two-dimensional gel electrophoretic (2D PAGE) analysis of the DNA synthesome associated PCNA isolated from nonmalignant and malignant breast epithelial cells. The results indicated that a unique form of PCNA was present in malignant breast cells. It was also determined that this

unique form of PCNA was not the result of growth stimulation or genetic alteration. This report describes the characterization of the unique form of PCNA in malignant breast epithelial cells, in nonmalignant breast epithelial cells transformed with oncogenes, and in serum obtained from a breast cancer patient.

METHODS

Cell culture: The malignant breast epithelial cell line (MCF 7) was cultured according to protocols provided by the American Type Culture Collection (ATCC). Briefly, cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum. The nonmalignant breast epithelial cells A1N4 and the transformed, nontumorigenic cell lines A1N4T and A1N4myc, were provided as cell pellets by Mark Lippman from the Lombardi Cancer Center (Georgetown University, Washington, DC).

DNA synthesome isolation: The DNA synthesome was isolated from MCF 7, A1N4, A1N4T, and A1N4myc cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for

3 min. The supernatant was removed and centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

Isolation of PCNA from serum: Serum collected from a patient with invasive intraductal breast carcinoma was Dounce homogenized and centrifuged at 2500 rpm for 10 min. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 0.1 mM PMSF) was added to the pellet and rocked at 4 °C for 2 hr. The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was collected and centrifuged at 60,000 rpm for 15 min. The supernatant was collected and used for analysis.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

[^{32}P] NAD$^+$ labeling: MCF 7 cells were washed twice with ice cold PBS and incubated in permeabilization buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, and 20 mM 2-Mercaptoethanol) on ice for 20 min. The cells were collected and washed in permeabilization buffer. Two hundred microliters of cells were added to 100 mM Tris-HCl, pH 7.8, 120 mM $MgCl_2$, 0.3 mM NAD$^+$ and 32 µCi ^{32}P NAD$^+$. The mixture was incubated at 30°C for 20 min. The reaction was stopped by placing the mixture on ice. The [^{32}P] NAD$^+$ labeled cells were Dounce homogenized and centrifuged at 12,000 rpm for 15 min in a microcentrifuge. The supernatant was collected for immunoprecipitation of PCNA.

Immunoprecipitation of PCNA: PCNA from MCF 7 cells and from [^{32}P] NAD$^+$ labeled MCF 7 cells were incubated overnight with an antibody directed against PCNA. Sixty µl of protein A conjugated agarose beads were added to the reactions and rocked for 1 hr. The reaction mixtures were washed twice with buffer A (0.154 M NaCl, 10 mM Tris-HCl pH 7.4, 0.05% Triton X-100, and 0.05% SDS) and three times with buffer B (0.154 M NaCl, 50 mM Tris-HCl pH 7.4, 2.5M KCl, and 0.5% Triton X-100). The protein was removed from the beads by incubation at 100°C for 30 sec and analyzed by both one dimensional and two dimensional gel electrophoresis.

One dimensional polyacrylamide gel electrophoresis (1D PAGE): Immunoprecipitated PCNA was loaded onto an 8% acrylamide SDS gel, and electrophoresed at 120 volts for 1 hr.

2-Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 µg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular mass. The proteins were then transferred electrophoretically to nitrocellulose membranes.

RESULTS

PCNA in Transformed Breast Cells

The cell line A1N4, a nonmalignant immortalized breast epithelial cell line, was transformed by the oncogenes c-myc and SV40 T antigen to establish two

stable cell lines: A1N4myc and A1N4T respectively. The A1N4, A1N4myc and A1N4T cell line are not tumorigenic in nude mice. 2D PAGE and Western blot analysis using an antibody against PCNA were performed. The A1N4 cell line did not contain the cancer specific acidic form of PCNA (Figure 2A). However, the A1N4myc and A1N4T cell lines did contain the altered form of PCNA (Figure 2B-C). Overexpression of the c-myc gene in A1N4 cells led to the expression of both forms of PCNA; while the transformation of the A1N4 cell line with SV40 T-antigen resulted in the expression of only the acidic form of PCNA.

PCNA in Serum

To determine whether the malignant form of PCNA could be a useful marker for identifying individuals with breast cancer, serum collected from a breast cancer patient was examined for the presence of the acidic form of PCNA. A serum sample collected from a breast cancer patient with stage III invasive intraductal breast carcinoma was analyzed by 2D PAGE and Western blot analysis using an antibody directed against PCNA. The Western blot analysis showed that the serum sample contained the altered form of PCNA (Figure 3). PCNA was not detected in control serum samples collected from two cancer free individuals. This result indicated that the cancer specific form of PCNA had been released into the peripheral blood from the tumor cells. Furthermore, the data indicate the nonmalignant cells do not release detectable levels of PCNA.

Post-translational Modification of PCNA

To understand the molecular basis for the different electrophoretic mobility of the two forms of PCNA, the protein was examined for differential post-translational modification. PCNA was examined to determine whether differences existed in the post-translational modification by poly(ADP-ribosylation) between the basic and cancer specific forms of the protein. MCF 7 cells were labeled with [^{32}P] NAD^{+}, the precursor for poly (ADP-ribose), and the

PCNA was immunoprecipitated from these cells. One dimensional PAGE analysis was performed to determine whether the immunoprecipitated PCNA was poly (ADP-ribosylated). Figure 4A illustrates that the immunoprecipitated PCNA was modified by ribosylation. 2D PAGE analysis was then performed to determine which form of PCNA was ribosylated. Figure 4B illustrates that the basic form of PCNA was ribosylated, while the cancer specific acidic form of PCNA was not modified by ribosylation.

DISCUSSION

PCNA plays a critical role in DNA replication and DNA repair. These two processes are involved in maintaining the integrity of the human genome and help to regulate cell proliferation. It has been shown that breast cancer cells contain a unique form of PCNA (Bechtel *et al.*, submitted). However, the underlying mechanisms resulting in the formation of the unique form of PCNA have not been elucidated. This study was designed to examine the unique form of PCNA in human breast cancer cells and in serum collected from a breast cancer patient.

The proto-oncogene c-myc and the oncogene SV40 T-antigen were introduced individually into the nonmalignant human breast epithelial cell line 184A1N4 (A1N4) to create two stable cell lines that overexpressed either c-myc (A1N4myc) or the SV40 T antigen (A1N4T). 2D PAGE analysis demonstrated that the A1N4myc and A1N4T cell lines contain the cancer specific acidic form of PCNA while the non-transformed parental cell line, A1N4, does not contain the acidic form of the protein. These findings suggest that following transfection of the c-myc or SV40 T-antigen gene cellular transformation events occurred in the cells resulting in the formation of the altered form of PCNA. This result is significant because up to 30% of breast cancers overexpress the c-myc gene

(Escort, 1986). The cancer specific form of PCNA may have a role in the tumorigenic process as evidenced by its presence in a nontumorigenic cell line overexpressing the c-myc gene, and also because alterations in the post-translational modification of PCNA may directly affect the DNA synthesis and repair processes in the cell. A decrease in the fidelity with which transformed cells replicate or repair DNA has been postulated to lead to the generation of genomic instability. This characteristic of tumor cells has been postulated to develop from a faulty DNA replication or repair apparatus (Loeb, 1991) and has been classified as a "mutator phenotype" expressed by transformed cells.

To be potentially beneficial as a tumor marker for the detection of breast malignancy, the altered form of PCNA should be readily detectable in the serum of a patient with breast cancer. In evaluating cancer development, the analysis of serum samples for specific tumor markers has failed to identify satisfactory markers to use for diagnosis and for monitoring the progress of patients with breast cancer (Hayes, 1996; Schwartz *et al.*, 1993). In general PCNA has not been very useful as a tumor marker for the prediction of patient outcome (Haerslev *et al.*, 1996; Schmitt *et al.*, 1994). The present study demonstrated that the altered form of PCNA can be readily detected in the serum collected from a patient with stage III invasive intraductal breast cancer, and that PCNA is not detectable in the serum from cancer free individuals. This finding suggests an intriguing possibility that serum testing for PCNA may be beneficial for the detection of residual disease or disease recurrence in breast cancer patients.

Previously, it was reported that the cancer specific form of PCNA in breast cancer cells did not result from either growth stimulation or genetic mutation within the PCNA gene (Bechtel *et al.*, submitted). The acidic form of PCNA is most likely the result of differential post-translational modification. It is known that PCNA is not phosphorylated and most likely it is not acetylated or

glycosylated (Bravo and Celis, 1985). Simbulan *et al.* (1996) reported that several components of the DNA synthesome, including PCNA, are modified by poly(ADP-ribosylation). In the present study, MCF 7 cells labeled with [^{32}P] NAD^+, the precursor of poly (ADP-ribose), were examined to determine whether PCNA was ribosylated. 2D PAGE analysis of the immunoprecipitated PCNA indicated that the basic form of PCNA was modified by poly(ADP-ribosylation), while the cancer specific acidic form of PCNA was unmodified. The difference in the ribosylation state between the two forms of PCNA may explain the charge difference between the acidic and basic forms. Although there has been no functional significance attributed to the ribosylation of PCNA, this post-translational modification may have a role in regulating the activity of the protein. The ribosylation state of PCNA may also affect its interactions with other proteins such as polymerase δ or $p21^{WAF1}$ which participate in the regulation of cell proliferation.

A characteristic which is common to virtually all types of cancer cells is aberrant cell proliferation. The aberrant proliferation of malignant cells appears to be due to failure of one or more of the regulatory mechanisms involved in the control of cell division. DNA replication is one of the critical regulatory processes that controls the rate of cell proliferation. Loss of function or alterations in proteins associated with DNA synthesis have been hypothesized to result in uncontrolled cell proliferation. The altered form of PCNA, a component of the DNA synthesome, was identified in breast cancer cells. The identification of the acidic form of PCNA in 100% of the breast cancer cells examined and its absence from benign tissue suggests that the acidic form of PCNA may contribute to the development of cancer. Further research into PCNA modifications are anticipated to provide new insight into its role in the tumorigenic process.

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Valverius, E.M., Bates, S.E., Stampfer, M.R., Clark, R., McCormick, F., Salomon, S.D., Lippman, M.E. and Dickson, R.B. (1989): Transforming growth factor alpha production and epidermal growth factor receptor expression in normal and

oncogene transformed human mammary epithelial cells. Mol. Endocrinol. **3**: 203-214.

FIGURE LEGENDS

Figure 1: Model of the DNA synthesome. The DNA synthesome is composed of two components. The first component, the core complex, is composed of proteins and enzymes which are tightly associated with one another throughout the fractionation procedure. These include polymerases α, δ, ε and poly (ADP-ribose) polymerase (PARP); helicases I & IV; DNA ligase I; RF-C; Ku; topoisomerase II; primase; DNA methyl transferase; and proliferating cell nuclear antigen (PCNA). The second component is the initiation complex which is responsible for the initiation events associated with replication. These proteins are more loosely associated with the core complex. These proteins include topoisomerase I and replication protein A (RPA).

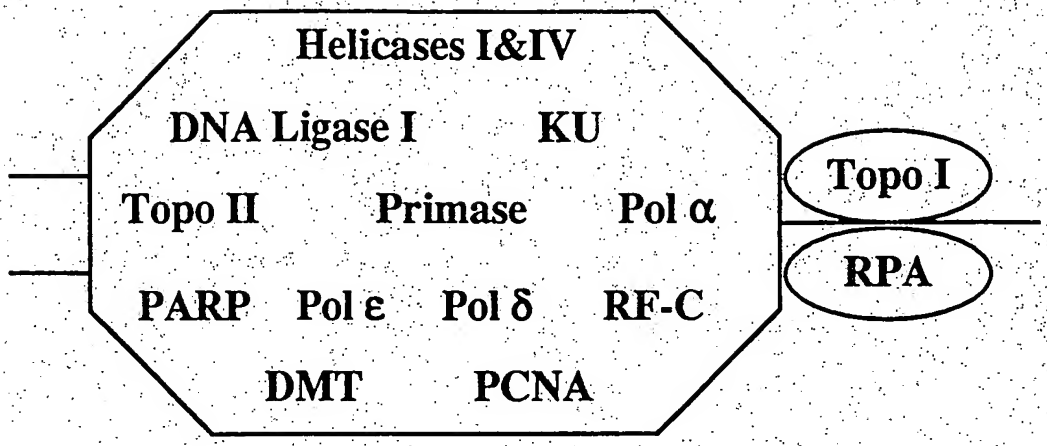
Figure 2: Protein migration pattern of PCNA from A1N4, A1N4myc, and A1N4T cells. The DNA synthesome was isolated from A1N4, A1N4myc and A1N4T cells and the components separated by 2D PAGE. Western blot analyses using an antibody directed against PCNA are shown for (A) A1N4 cells (B) A1N4myc and (C) A1N4T cells. The arrow indicates the position of the altered form of PCNA.

Figure 3: Protein migration pattern of PCNA from serum. A serum sample from a patient with invasive intraductal breast cancer was subjected to 2D PAGE and Western blot analysis using an antibody specific for PCNA. The arrow indicates the position of the altered form of PCNA.

Figure 4: PCNA is poly (ADP-ribosylated). MCF 7 cells were labeled with [^{32}P] NAD^+. PCNA was immunoprecipitated from the labeled cells and analyzed

by one dimensional and two dimensional PAGE. (A) one dimensional analysis (B) 2D PAGE analysis.

Figure 1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Primase
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Figure 2

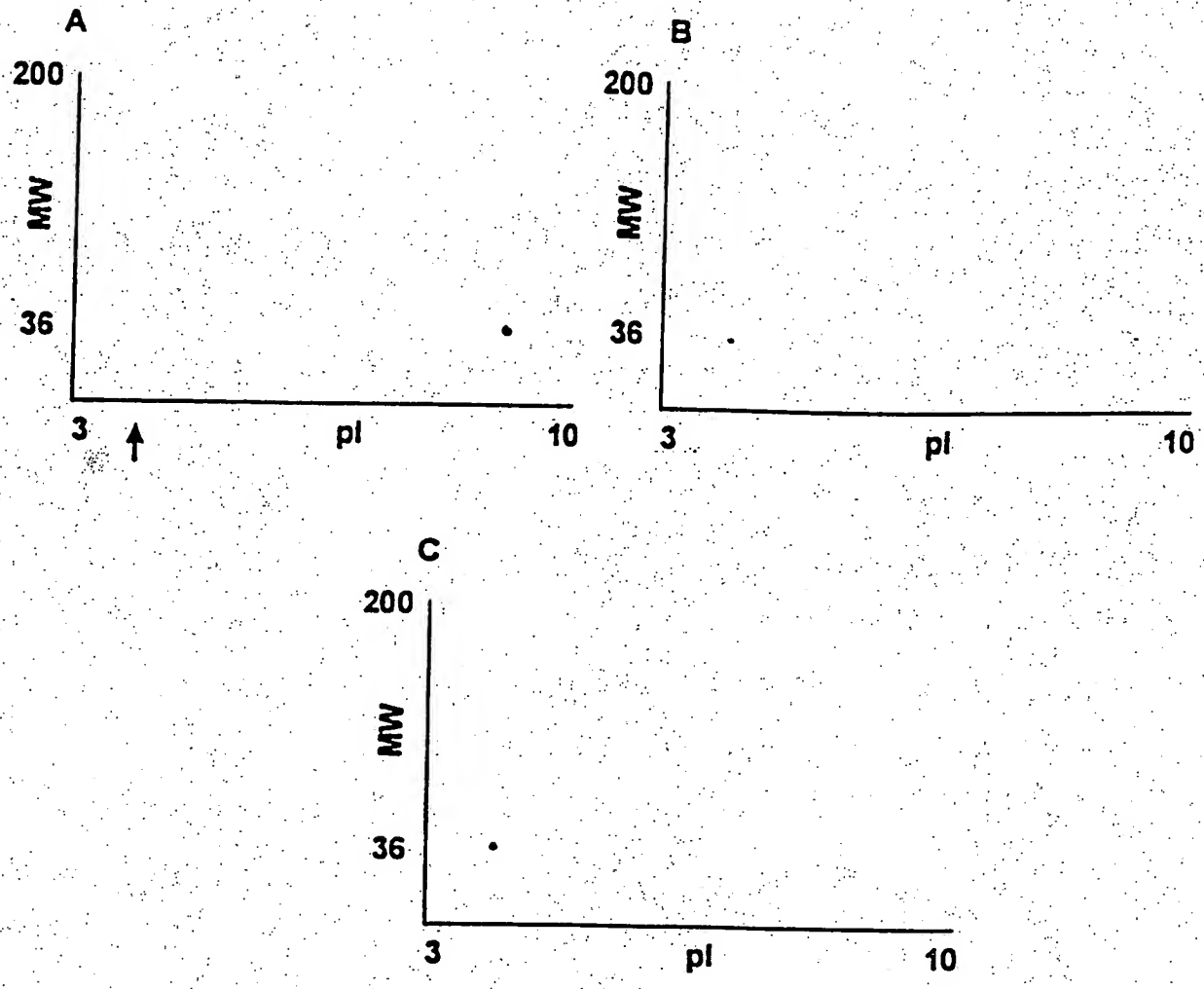


Figure 3

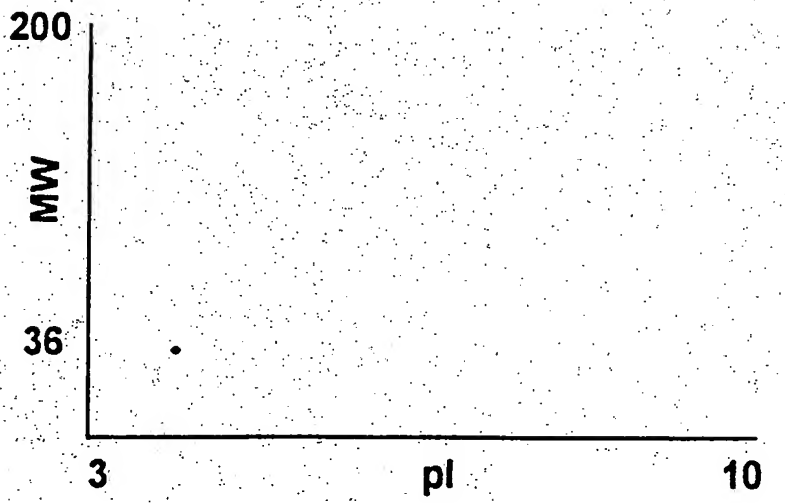
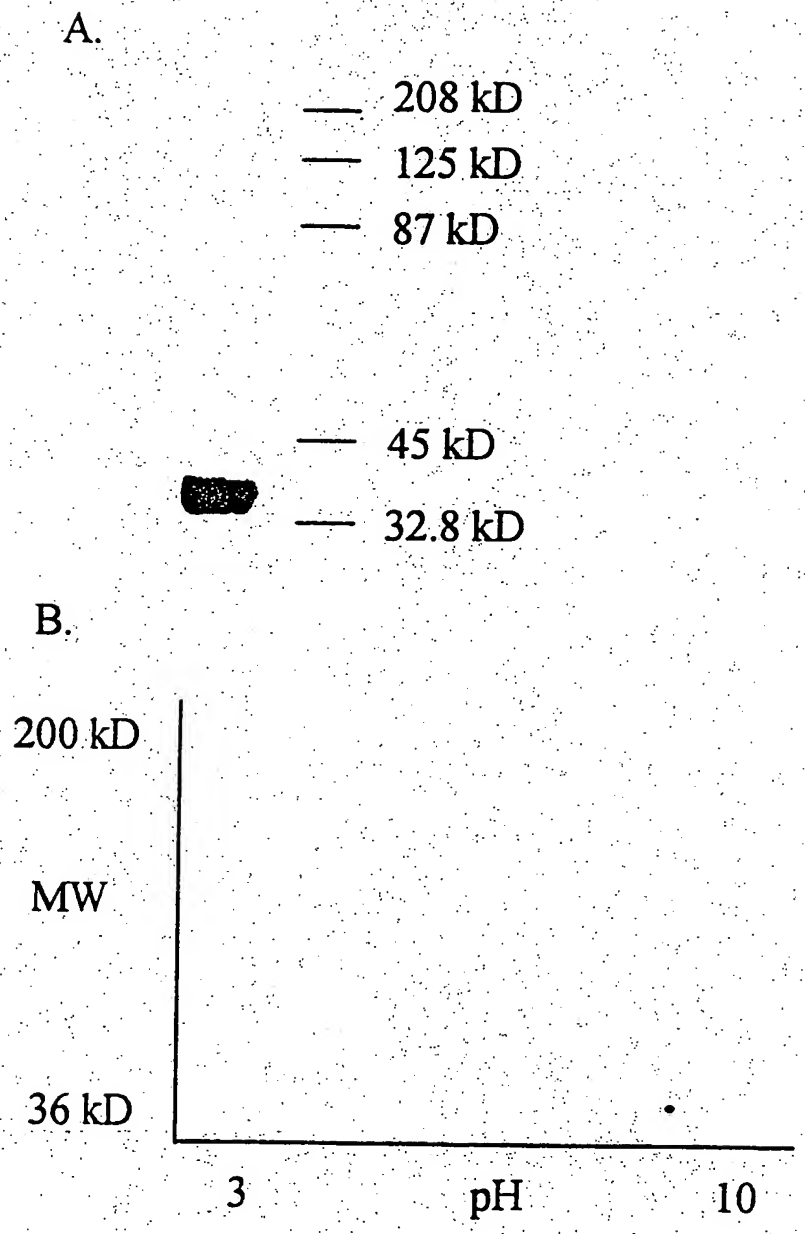


Figure 4



Chapter IV

Title: An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers.

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Key Words: PCNA, tumor marker, post-translational modification, epigenetic change, breast cancer, prostate cancer, malignant glioma, leukemia, cervical cancer, colon cancer

ABSTRACT

The process transforming benign cells into malignant cells involves a complex series of molecular and structural alterations which are poorly understood. One characteristic common to most malignancies is the aberrant proliferation of cells. Cellular proliferation is regulated at a number of points throughout the cell cycle through complex interaction of many proteins. Together these proteins form checkpoints that restrict the ability of cells to progress through the cell cycle. Several critically important checkpoints monitor the cells prior to the entrance into S phase. These checkpoints ultimately signal the DNA synthetic apparatus to begin DNA replication. A multiprotein DNA replication complex, DNA synthesome, has been isolated and characterized from human cells and found to be fully competent to mediate DNA replication *in vitro*. Proliferating cell nuclear antigen (PCNA) was found to be a component of the DNA synthesome. PCNA is an accessory protein for polymerase δ and is involved in DNA repair. A recent study has identified a unique form of PCNA in malignant breast cells that significantly differs in isoelectric point (pI) from the PCNA found in nonmalignant breast cells. The purpose of this study was to determine whether different types of malignant cells contain the altered form of PCNA. To examine this possibility, the DNA synthesome was isolated from malignant prostate cells (LNCaP, PC50), malignant colon cells (KGE90, KYE350, SW48), cervical cancer cells (HeLa), malignant brain cells (T98) and leukemia cells (HL60, CML, AML). The components of the synthesome were resolved by 2 dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA. The results of the analysis demonstrated that the altered form of PCNA was present in all of the malignant cells examined. These findings suggest that the structural alteration of PCNA

may be involved in the development of malignancies due to the critical role PCNA has in the DNA replication and repair processes.

INTRODUCTION

Cancer is the second leading cause of death in the United States (Parker *et al.*, 1997). Lung cancer alone was responsible for more than 160,000 cancer related deaths in 1997, and it is the leading cause of cancer mortality (Parker *et al.*, 1997). Among men, prostate cancer is the second leading cause of cancer related deaths and more than 300,000 new cases were diagnosed in 1997 (Parker *et al.*, 1997). Breast cancer is the second leading cause of cancer related deaths among women and approximately 180,000 new cases of breast cancer were diagnosed in 1997 (Parker *et al.*, 1997). Colon cancer is also a leading cause of cancer deaths in men and women, with 46,000 deaths attributed to this malignancy in 1997 (Parker *et al.*, 1997; Weinberg and Strom, 1995). Leukemias and brain cancers are the most common childhood malignancies with acute lymphocytic leukemia accounting for 80% of childhood cancers (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The origin and clinical manifestations of a malignancy differ for each individual cancer. Breast cancers are usually ductal or lobular in origin and generally affect post menopausal women (Dickson and Lippman, 1992). Prostate and colon cancers generally arise from benign lesions which progress into malignancies. Usually these forms of cancer affect people over the age of fifty (Isaacs, 1997; Meyers and Grizzle, 1997; Parker *et al.*, 1997; Johnson, 1995; Weinberg and Strom, 1995). Many brain tumors arise from astrocytes and are generally refractory to chemotherapy (Kordek *et al.*, 1996; Dalrymple *et al.*, 1994). Cervical cancer has been shown to be caused by human papillomavirus (HPV), and generally occurs in women over the age of thirty five (Villa, 1997; Parker *et al.*, 1997). Leukemias, in contrast to solid tumors, arise in the bone marrow and result in the circulation of immature white cells in the peripheral blood (Kumar *et al.*, 1992). Leukemia can affect people of all ages but children

with leukemias have a significantly increased survival rate compared to adults (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The diagnosis of cancer at an early developmental stage is important in reducing cancer mortality. The use of mammography to detect developing breast cancers has reduced the mortality rate of the disease (Hayes, 1996). Testing for the presence of prostate specific antigen (PSA) has enabled clinicians to diagnose prostate cancer in men early in its onset (Small, 1997; Gao *et al.*, 1997). As a marker, PSA has high sensitivity and specificity and correlates with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). However, a major limitation is the inability to distinguish between benign and malignant prostate lesions by evaluating PSA (Pandha and Waxman, 1995; Magdelenat, 1992). Routine Pap smear screens have been routinely used for the past twenty years and can be beneficial for the early detection and monitoring of cervical cancer among women. Although screening methods exist to detect certain types of malignancy the basic mechanisms for the development of cancer have not been determined.

One commonality of virtually all malignancies is the aberrant proliferation of cells. Aberrant cell proliferation is presumably due to escape from the normal controls which regulate the progression of the cell into specific stages of the cell cycle. Recently, an altered form of the protein proliferating cell nuclear antigen (PCNA) was identified from malignant human breast cells (Bechtel *et al.*, submitted). PCNA is a nuclear protein that is required for DNA synthesis and DNA repair. In this report, PCNA was analyzed from a variety of human malignancies to determine whether the unique form of PCNA was present only in malignant breast cells or whether it was present in other types of malignant cells. Several malignant human cell lines were used: LNCaP and PC50 (prostate adenocarcinoma); KGE90, KYE350 and SW48 (colon adenocarcinoma); T98

(malignant glioma); HeLa (cervical carcinoma); and HL60 (promyelogenous leukemia). PCNA was also analyzed from chronic myelogenous leukemia specimens, acute myelogenous leukemia sample and serum collected from cancer free individuals.

METHODS

Cell culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated from LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and

centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

Analysis of prostate cancer cells in this study demonstrated the presence of the altered form of PCNA. Previous reports indicated that PCNA is strongly expressed in prostate cancers and also has been detected in dysplastic luminal cells of intraepithelial neoplasia, a premalignant prostate lesion (Pandha and Waxman, 1995; Magdelenat, 1992). Gao *et al.* (1997) reported that PCNA levels correlate with the clinical stage and metastatic state of prostate tumors. The researchers suggested that PCNA levels have the potential to be used as a prognostic marker for prostate cancer (Gao *et al.*, 1997).

The colon cancer cell lines examined in this study also contained the altered form of PCNA. Studies have shown that PCNA labeling index is correlated with tritiated thymidine uptake in assessing the proliferative activity of colon tumors (Bleiberg *et al.*, 1993). Neoptolemos *et al.* (1995) demonstrated that PCNA levels used in conjunction with the Duke's classification system (the classification system for colon tumors based on histological examination) improved the prediction of survival for colon cancer patients. Mitchell *et al.* (1997) demonstrated that increased levels of PCNA are found in primary colon tumors and in resulting lung metastases. It has been shown that PCNA levels increase during the progression of benign lesions to malignancy and that increased levels are associated with the recurrence of adenomas (Shpitz *et al.*, 1997; Paspatis *et al.*, 1994; Risio *et al.*, 1993). Risio *et al.* (1993) hypothesized that the increase in the number of PCNA labeled cells is related to hyperproliferation as well as to the neoplastic deregulation of PCNA synthesis in the development of colon cancer.

Malignant gliomas are composed of heterogeneous subpopulations of cells with different cytogenetic compositions making it difficult to identify useful prognostic markers (Dalrymple *et al.*, 1995). Previous studies demonstrated that PCNA labeling index correlates with the histological grade of the brain

malignancy. In the majority of benign astrocytomas, the PCNA labeling index is low (Kordek *et al.*, 1996). Kordek *et al.* (1996) also have hypothesized that malignant gliomas contain two forms of PCNA, one of which does not have a role in DNA synthesis. The results of the present study have indicated that malignant glioma cells contain the altered form of PCNA. The functional role of the altered form of PCNA needs to be examined in greater detail to determine whether it has a role in the development and progression of malignant gliomas.

The role of PCNA in the development and progression of CML is not well characterized. CML is a biphasic disease characterized by an early chronic phase followed by a blast phase (Zaccaria *et al.*, 1995). Takasaki *et al.* (1984b) demonstrated a correlation between the number of leukocytes expressing PCNA and the percent of blast cells in blood during the blast phase of CML. These investigators also identified the presence of non-blast cells which were positive for PCNA in the peripheral blood during the blast phase of CML. This result differs from the observation that the non-blast cells were negative for PCNA in the chronic phase (Takasaki *et al.*, 1984). The PCNA labeling index for CML cells is not significantly different form normal bone marrow cells (Thiele *et al.*, 1993). However, in the chronic myeloid proliferative disorder osteomyelofibrosis, there is a significant increase in the PCNA labeling index (Thiele *et al.*, 1994). Interferon treatment resulted in decreased PCNA labeling. In the present study, the results demonstrated that the leukemia samples examined contain the altered form of PCNA, while samples collected from cancer free individuals did not contain the altered form of PCNA.

Analysis of HeLa cells in the present study revealed that these cervical cancer cells contain the altered form of PCNA. Cardillo *et al.* (1993) found that PCNA levels were increased in all cases of invasive squamous carcinoma, cervical adenoma, cervical carcinoma in situ (CIS) and cervical intraepithelial

neoplasia (CIN) III examined. Smela *et al.* (1984) demonstrated a significant correlation between PCNA index and the severity of cervical dysplasia by determining the PCNA levels in CIN I, CIN II and CIN III lesions. Other studies found that PCNA index was increased in premalignant and malignant cervical lesions compared to nonmalignant tissue (Steinbeck *et al.*, 1995; Raju *et al.*, 1994; Shurbaji, 1993). Kobayski *et al.* (1994) concluded that the increase in PCNA labeling index suggests that there is considerable alteration of biologic activity, including genetic mutations, during the progression of severe cervical dysplasia to CIS.

This study examined several human malignancies for the presence of the acidic form of PCNA. The malignancies examined in this study have few common characteristics or clinical manifestations. Previously, an altered form of PCNA was identified in malignant breast cells. It was demonstrated that the unique form of PCNA was not the result of growth stimulation or genetic mutation (Bechtel *et al.*, submitted). Further research indicated that differential post-translational modification by poly(ADP-ribosylation) was at least partially responsible for the presence of the altered form of the protein (Bechtel *et al.*, submitted). This report identified the acidic form of PCNA in leukemia, prostate, cervical, colon and brain cancers. These data demonstrated a simple molecular alteration in the PCNA polypeptide is a common feature between these diverse types of cancer. These findings suggest that the acidic form of PCNA may have an underlying role in the development of different types of cancer and that these cancers may develop as a consequence of a global alteration in the manner in which specific cellular proteins are post-translational modified.

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FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1

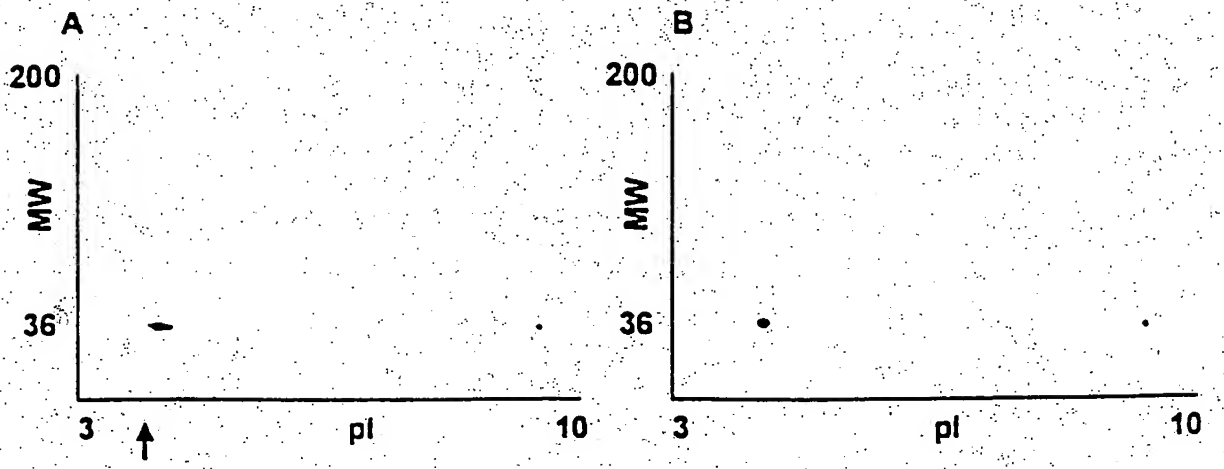


Figure 2

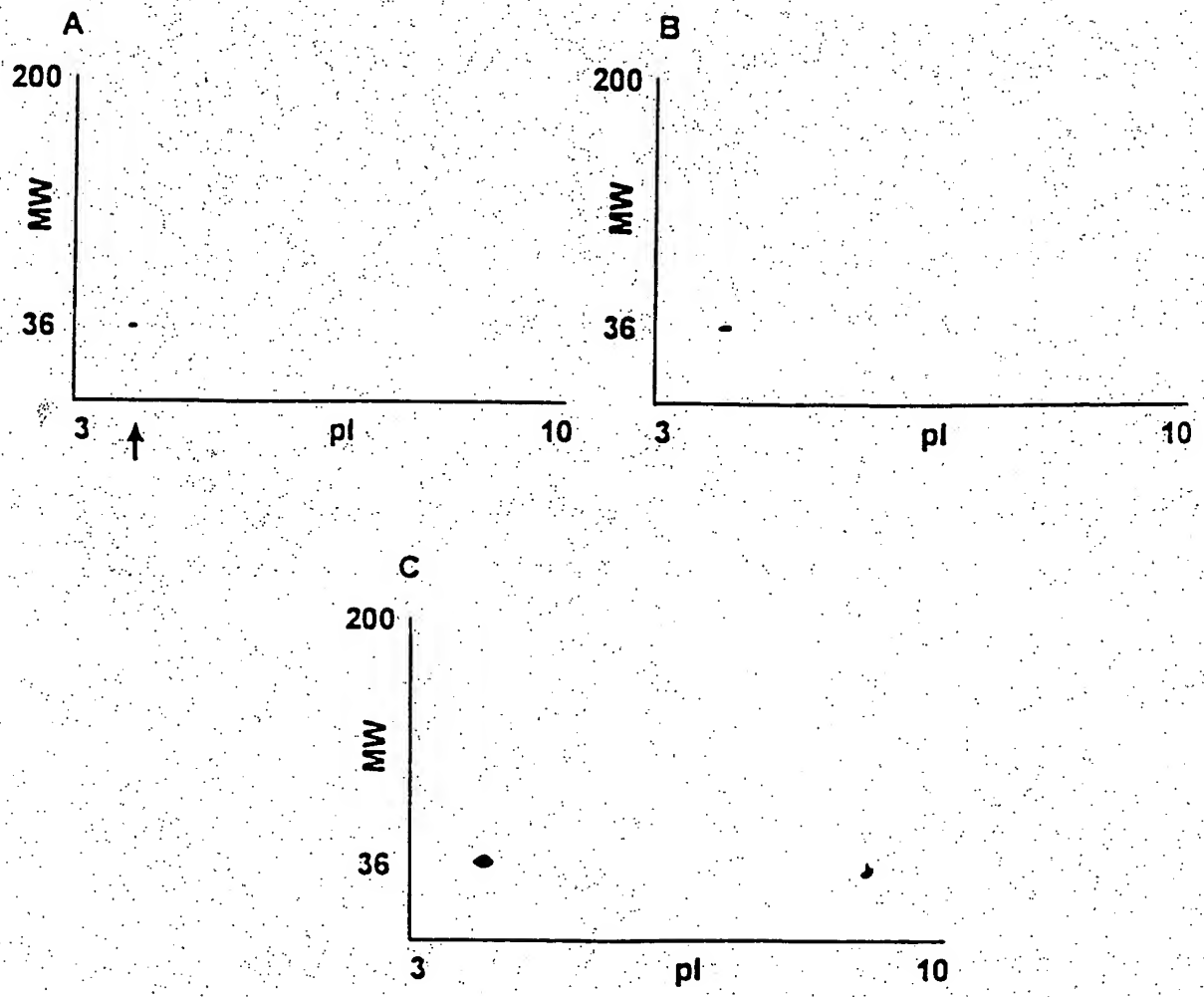


Figure 3

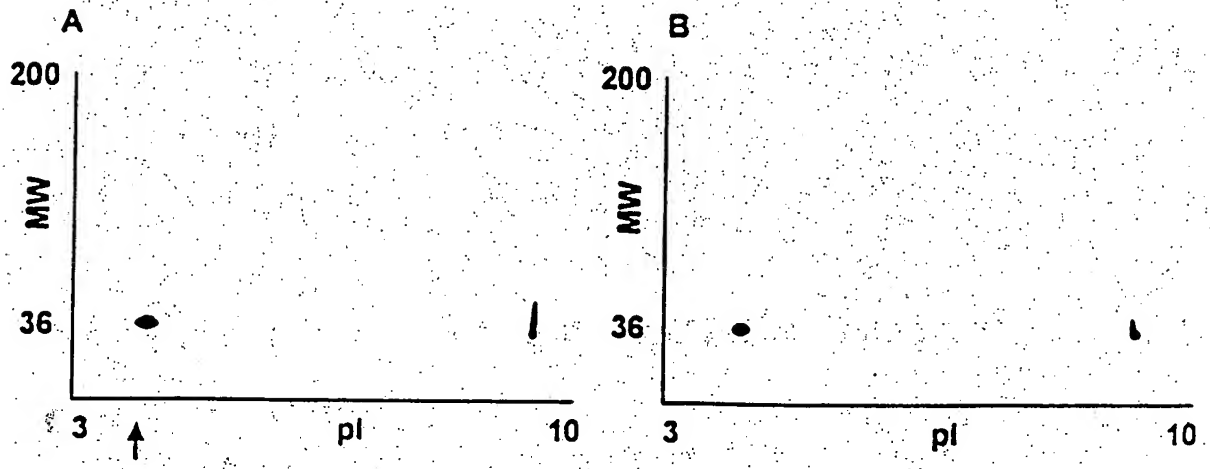
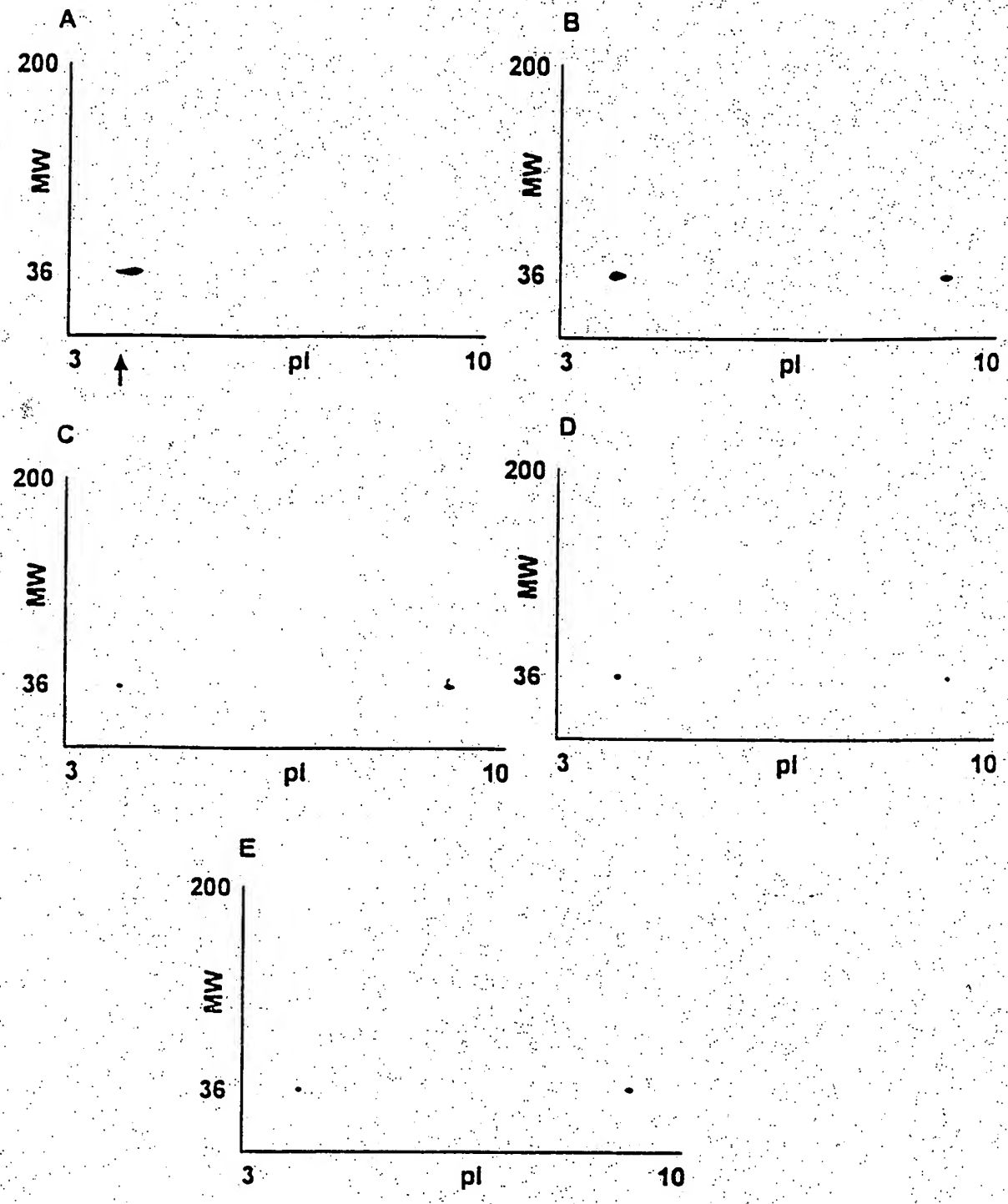


Figure 4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Chapter IV

Chapter IV

Schleicher & Schuell Application for Post-Doctoral Fellowship

Dear Post-Doctoral Fellowship Applicant:

Thank you for your interest in the Schleicher & Schuell Post-Doctoral Fellowship. In this packet you will find specific instructions on applying for the fellowship, rules and regulations, and release forms that are required for eligibility.

In preparing the application materials, use English and avoid jargon. For terms not universally known, spell out the term in the first instance with the appropriate abbreviation in parenthesis. The abbreviation or acronym may be used thereafter.

Application materials must be prepared single sided and single spaced. Use standard size, black text and prepare all charts, diagrams, and tables in black ink.

The following information must be provided to be eligible for the fellowship:

1. Academic Profile. (Degrees earned, colleges attended, scholarships, awards received.).
2. Related Research/Work Experience.
3. Description of Research, 4 to 8 pages.
4. A Letter of Support From Prospective Mentors.
5. Mentor's *curriculum vitae.*
6. Research approvals from individual mentor, department chairman, and institution.

Thank you for applying for the Schleicher & Schuell Fellowship, and Good Luck!

Sincerely,

David L. Reilly
Director Life Science

REVISED

Schleicher & Schuell Post Doctoral Fellowship Rules and Regulations

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2. Research is confined to basic research relating to the molecular biology of breast cancer and/or methods of identification of individuals with a predisposition to develop breast cancer.

3. Applications must be received by ~~May 1, 1998~~ ***July 1, 1998***. Applications must be in English and avoid jargon, use standard size black text, and all diagrams and charts must also be produced in black. Specific application instructions are included in the attached form.

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The post-doc fellowship will provide a salary and a small travel stipend for a maximum of two years. Funding will be $25,000 per year, with total funding not to exceed $50,000.

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All applications will be reviewed by a panel of internal investigators. The fellowship will be awarded on ~~**June 15, 1998**~~ **August 15, 1998.** Applications will be treated in a confidential manner. The Schleicher & Schuell post-doctoral fellowship is an equal opportunity fellowship and will be awarded regardless of gender, race, or religion.

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PC WordPerfect, or in PC Microsoft Word, also suitable for the Mac.

The deadline for applications to be received is ~~**May 1, 1998**~~ Deadline extended to **July 1, 1998**.

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Chapter V

Discussion

One of the hallmarks of malignancy is the accumulation of genetic mutations which contributes to genetic instability exhibited by many types of cancer cells. Some of these mutations are postulated to contribute to the uncontrolled cellular proliferation observed for most tumors. The accumulation of genetic errors in cancer cells is relatively high; considering that nonmalignant cells are estimated to make only 1.4×10^{-10} mutations/ base pair/ cell division (Cheng and Loeb, 1993; Loeb, 1998). This observation has to the hypothesis that transformed cells develop a "mutator phenotype" (Loeb, 1991). Following the initial work reported by Sekowski *et al.* (submitted) that the DNA replication apparatus of malignant breast cells was mutagenic, this study was designed to identify whether structural differences in specific DNA replication proteins exist between malignant and nonmalignant cells. In this study an altered form of PCNA was identified in malignant cells.

PCNA was first identified by an autoantigen found in the serum of lupus (SLE) patients. This autoantigen was the first antibody found to react solely in the nuclei of proliferating cells. Miyachi *et al.* (1978) identified PCNA in cells from lymph nodes, spleen and thymus as well as in rapidly proliferating cell lines. They also noted that PCNA was absent from cells which did not proliferate. These investigators hypothesized that PCNA might be useful as a marker to identify proliferating cells. Takasaki *et al.* (1984a) purified PCNA from a rat thymus extract. Analysis of the purified protein demonstrated that the antigen was present in the nucleus of cells, and that it had an isoelectric point (pI) of 4.8. These investigators also noted the presence of PCNA in blast cells found in the peripheral blood of CML patients. Mathews *et al.* (1984) identified PCNA in human malignant cervical cells (HeLa cells) and suggested that the protein may be involved in cell division and/or the transformation of cells. Bravo and Graf

(1985) found that PCNA synthesis directly correlated with cell proliferation by comparing the levels of PCNA in the cell with tritiated thymidine uptake. The investigators suggested that PCNA was involved in DNA replication. Celis and Celis (1985) examined synchronously growing transformed human amnion cells (ANA) with an antibody specific for PCNA. The results demonstrated that cells in the G_1, G_2, and mitosis phases of the cell cycle contained little PCNA. PCNA increased and reached maximum levels during S phase suggesting that PCNA played a role in DNA replication. Celis and Madsen (1986) studied PCNA in UV irradiated ANA cells and found an increase in PCNA levels in non S phase cells. The investigators suggested that PCNA may also be involved in nucleotide excision repair. Tan *et al.* (1986) then identified an auxiliary protein for DNA polymerase δ from calf thymus. This protein coelutes with polymerase δ and is a single 37 kD band on an SDS gel. The protein did not have DNA polymerase, DNA primase, ATPase or nuclease activity. The protein increased polymerase δ binding to a DNA template and was identified as PCNA.

The human gene encoding PCNA has been cloned (Almendral, 1987; Travali *et al.*, 1989). The gene is present in a single copy and codes for 262 amino acid protein containing 6 exons. The protein contains 41 acidic and 26 basic residues (Travali *et al.*, 1989). The gene contains extensive sequence similarities among introns and between introns and exons (Travali *et al.*, 1989). Comparison of the human protein sequence with the PCNA sequence from rat, drosophila and Saccharomyces cerevisea demonstrated an extensive homology between species (Shipman-Appasamy *et al.*, 1991). X-ray crystallography revealed that PCNA forms a trimer with a ring-like structure (Krishna *et al.*, 1994). There is a central channel lined with 12 α helices which is proposed to interact with DNA (Krishna *et al.*, 1994).

The PCNA gene and its 5' and 3' flanking regions were studied to determine whether the expression of the gene is autoregulated. The 5' flanking region was found to contain RNA polymerase II promoter activity (Travali *et al.*, 1989). Pietrkowski *et al.* (1991) demonstrated that mutations in the 5' flanking region of the PCNA gene resulted in increased levels of PCNA being expressed in growth arrested cells. They further demonstrated that serum stimulated increase in PCNA levels was independent of the 5' flanking region. Using gel shift assays it was shown that nuclear proteins bind to this 5' region of the gene. Ottavio (1990) demonstrated that intron 4 negatively regulated PCNA transcription. Using deletions in intron 4 the investigators showed that in growth arrested cells an increase in abundance of mRNA encoding PCNA was found relative to the levels seen in wild type cells (Ottavio, 1990). Alder *et al.* (1992) demonstrated that a 35 base pair region of intron 1 was a negative regulator of PCNA transcription. Mutations in this region resulted in increased PCNA mRNA levels in a thymidine kinase deficient temperature sensitive fibroblast cell line (TKts13) growth arrested at G_1. These data indicated that the PCNA gene was regulated at the level of transcription by multiple mechanisms.

PCNA transcription is affected by various cellular factors. Almendral *et al.* (1987) treated quiescent mouse 3T3 cells with hydroxyurea to create a block at the G_1/S boundary causing the inhibition of DNA synthesis. PCNA mRNA levels increased despite the inhibition of DNA replication, demonstrating that the increase in PCNA level is independent of DNA synthesis (Almendral *et al.*, 1987). Shipman-Appasmy *et al.* (1990) used a murine T helper cell line to demonstrate that interleukin 2 stimulates PCNA transcription and also stabilizes PCNA mRNA. Perry and Tindall (1996) studied the effect of androgens on PCNA transcription. Using LNCaP cells (a prostate cancer cell line) these investigators showed that androgens did not increase PCNA mRNA levels but that the androgens

increased the stability of the mRNA. These data further suggest that PCNA gene transcription is regulated at multiple levels through a variety of cellular mechanisms.

Since it was first detected in the serum of SLE patients, PCNA has been associated with cell proliferation (Miyachi *et al.*, 1978; Takasaki *et al.*, 1984a,b). Kurki *et al.* (1986) used nonmalignant spleen cells and the MOLT-4 leukemia cell line to show that PCNA expression is highly cell cycle dependent. These researchers noted that the levels of PCNA were low in early G_1 cells, started to increase in late G_1 and attained maximal levels during S phase. Levels of PCNA rapidly declined during the G_2/M phase of the cell cycle. Morris and Mathews (1989) studied PCNA levels in synchronized HeLa cells. In their study PCNA synthesis increased 2-3 fold during the S phase peak. Bolton *et al.* (1992) studied PCNA expression in synchronized CHO-K1 cells. Expression of PCNA during G_0 was depressed compared to asynchronously growing cultures. Levels of PCNA increased during mid G_1; reaching peak levels in S phase. Bravo and MacDonald-Bravo (1985) examined PCNA levels in hydroxyurea treated 3T3 cells. The hydroxyurea blocked the cell cycle at the G_1/S boundary preventing DNA replication. Treatment with hydroxyurea did not prevent the levels of PCNA from increasing during late G_1. However, the characteristic decrease in PCNA levels that occurs during late S phase was prevented, suggesting that events occurring during S phase regulate PCNA levels.

An auxiliary factor for polymerase δ isolated from calf thymus was identified as PCNA (Tan *et al.* 1986). PCNA functioned to stabilize polymerase δ on the primer template allowing longer replication products to be synthesized (Tan *et al.*, 1986). Studies have shown that PCNA specifically stimulates polymerase δ and does not affect the activity of polymerase α. Zuber *et al.* (1989) examined the role of PCNA in DNA replication using unfertilized eggs of

Xenopus laevis on the replication of injected plasmid. The addition of an anti-PCNA antibody resulted in a 67% decrease in DNA replication. By adding anti-polymerase α and anti-PCNA antibodies DNA replication was completely inhibited. These results demonstrated that PCNA interacts with polymerase δ and that both polymerases α and δ are required for DNA replication. Melendy and Stillman (1991) examined the effect of PCNA antibody on SV40 DNA synthesis using a crude HeLa cell extract. The anti-PCNA antibody inhibited the formation of long DNA replication products, suggesting that PCNA is involved in leading strand DNA synthesis. Lee and Hurwitz (1990) examined the interaction of PCNA and polymerase δ with two other replication proteins, (i.e. single stranded DNA binding protein (RP-A) and activator 1 (RFC)), on the elongation of a primed template. It was observed that the proteins formed a stable complex with the DNA in an ATP dependent manner. PCNA has been identified as a component of a multiprotein DNA replication complex. This complex, the DNA synthesome, was first isolated and characterized from HeLa cells by Malkas *et al.* (1990). The synthesome is composed of all the proteins necessary for DNA replication, and is fully competent to mediate SV40 *in vitro* DNA synthesis. The addition of anti-PCNA antibody to the *in vitro* replication assay with the DNA synthesome resulted in a decrease in replication activity, but did not completely abolish replication. PCNA was also identified as a component of the synthesome through Western blot analysis. All of these data indicate that PCNA acts as an auxiliary protein for polymerase δ during DNA replication.

In 1986 Celis and Madsen examined PCNA in ultraviolet (UV) irradiated ANA cells. The conditions induced nuclear excision repair synthesis in the UV treated cells. The investigators found an increase in the number of non-S phase cells that were labeled with an anti-PCNA antibody. Treatment of the cells with cyclohexamide, an inhibitor of protein synthesis, did not affect the increase in

labeled cells following UV treatment. These results suggested that the increase in PCNA staining was due to a redistribution of pre-existing PCNA and that PCNA was involved in nucleotide excision repair. Toschi and Bravo (1988) examined PCNA in UV irradiated noncycling human fibroblasts. The investigators found that increases in PCNA (detected by indirect immunoflurescence staining) correlated with both the UV dose that cells received and with the DNA repair synthesis activity of the cells. Using an assay system that measures the excision and repair of thymidine dimers, Nichols and Sancor (1992) demonstrated that PCNA was not involved in the excision step of the repair process, suggesting that PCNA is involved in the resynthesis step of DNA repair. Shioji *et al.* (1992) devised a system in which stable DNA intermediates were formed from UV treated HeLa cells. Their results indicated that PCNA was required for the synthesis and ligation steps during the completion of DNA repair events. Umar *et al.* (1996) used a mismatch template to demonstrate that DNA repair synthesis could be inhibited by $p21^{WAF1/clp1}$ (p21, which binds to PCNA and inhibits the ability of PCNA to participate in cellular processes) indicating that PCNA is necessary for this repair process.

In the cell PCNA interacts with various proteins. During DNA replication PCNA interacts with replication factor C (RFC) and polymerase δ to mediate leading strand DNA synthesis (Lee and Hurwitz, 1990; Melendy and Stillman, 1991). RFC is composed of 5 polypeptides and Pan *et al.* (1993) demonstrated that the 40 kD RFC polypeptide directly interacted with PCNA during replication. Flores-Razos *et al.* (1994) used the surface plasmon resonance technique and gel filtration to demonstrate the direct interaction of p21 and PCNA. The protein p21 apparently inhibits that activity of PCNA during DNA replication. Polymerase δ mediated DNA replication was inhibited by the addition of p21 and analysis of the replication products revealed that the formation of long replication products

was inhibited (Flores-Razos *et al.* 1994). Gulbis *et al.* (1996) demonstrated through X-ray crystallography that p21 binds to PCNA in a 1:1 stoichiometry and suggested that p21 inhibited PCNA activity by masking those functional groups on the PCNA trimer which are required for interaction with polymerase δ or RFC. The interaction of p21 and PCNA is different during nucleotide excision repair in UV irradiated cells. Liu *et al.* (1996) demonstrated that UV irradiation resulted in an increase in the expression of p21 while it induced PCNA dependent nucleotide exsion repair. These results suggest that the PCNA involved in DNA repair events is not inhibited by p21; unlike the effect of PCNA involved in DNA replication.

Although it has been shown conclusively that PCNA is involved in both DNA replication and DNA repair there are questions as to whether PCNA has additional functions within the cell. Cyclin D1 is a cell cycle regulatory protein which appears to have a role in the progression of cells through the G_1 and S phases of the cell cycle. Xiong *et al.* (1992) demonstrated using coimmunoprecipitation and proteolytic digests that cyclin D1 interacts with PCNA. The functional relevance of this interaction is not known. Bravo and MacDonald-Bravo (1987) found that the location of PCNA in the nucleus changes during the course of S phase and that the relocation events could be blocked with DNA synthesis inhibitors. The investigators demonstrated through immunoflorescence staining that two populations of PCNA existed in 3T3 cells during S phase. One population was observed throughout the nucleus and was easily extracted using Triton X-100. The second population of PCNA was tightly associated with nuclear structures and resisted Triton X-100 extraction. Morris and Mathews (1989) demonstrated in HeLa cells that although PCNA synthesis increased during S phase, the proportion of PCNA in a cell remains constant during the cell cycle. Liu *et al.* (1995) observed that PCNA mRNA was stable and remained

constant throughout the cell cycle. Morris and Mathews (1989) also observed that a maximum of one-third of the PCNA in a cell is tightly associated with structures within the nucleus. These data imply that PCNA has additional roles within a cell besides DNA replication and DNA repair. These data also raise questions about why the mRNA encoding PCNA is present during the cell cycle in a constant amount whereas the PCNA polypeptide is usually observed only during S phase.

The data presented in this dissertation suggested that of two forms of PCNA exist in malignant cells. It was confirmed that two forms of PCNA were found in mailgnant cells and that these forms differed significantly in pI. Examination of malignant and nonmalignant breast cells revealed that the acidic form PCNA was found exclusively in malignant breast cells. The acidic form of PCNA was found in 100% of malignant breast tumors examined, which makes its use as a marker for malignant transformation significantly better than the currently used tumor markers (i.e. Her2/neu or BRCA1). Further examination determined that the acidic form of PCNA was not the result of growth stimulation. Genetic analysis revealed that the coding region of the PCNA gene from malignant and nonmalignant breast cells was identical and had not sustained mutations. This suggests that the acidic form of PCNA is the result of an epigenetic change. The results indicate that the acidic form of PCNA has the potential to serve as a marker for breast cancer.

The data suggested that the acidic form of PCNA most likely resulted from differential post-translational modification of the polypeptide between the malignant and nonmalignant cells. Bravo and Celis (1985) examined PCNA isolated from HeLa cells by 2D PAGE. The results showed that PCNA was not phosphorylated. They concluded that PCNA was not modified by acetylation, glycosylation or other modifications that would affect the charge of the protein.

Sadie and Mathews (1986) examined PCNA polypeptide produced using the cell free translation of HeLa mRNA. They found that the product of the translation reaction was a single polypeptide with the same molecular weight of the PCNA labeled in vivo. This result suggests that the PCNA was not derived from a larger precursor protein, and that it was not grossly modified by post-translational modifications. Simbulan *et al.* (1996) examined the DNA synthesome isolated from HeLa cells. The researchers demonstrated that PCNA was poly(ADP-ribosylated). In the present study the two forms of PCNA were examined for modification by poly(ADP-ribose), which is in contrast to previous findings. Metabolic labeling of MCF 7 cells demonstrated that the basic form of PCNA was ribosylated while the acidic form, found in malignant cells, was unmodified. The results suggest that differential poly(ADP-ribosylation) may partially account for the difference in charge between the two forms of PCNA. The functional significance of the differential post-translational modification of PCNA polypeptide has not been defined.

There is a strong possibility that post-translational modification by poly(ADP-ribose) may modulate either the level of activity or fidelity of the DNA replication process. Poly(ADP-ribose) polymerase (PARP) is responsible for the addition of ribose moieties to proteins. PARP activity has been shown to inhibit SV40 *in vitro* DNA replication (Eki, 1994). This enzyme inhibits the individual activities of DNA polymerases isolated from HeLa cells. This inhibition is intensified by the addition of NAD, which is the precursor for poly (ADP-ribose). These data suggest that poly (ADP-ribosylation) of the DNA polymerases may act as a negative regulator of DNA replication. The basic and acidic forms of PCNA need to be assessed to determine whether poly (ADP-ribosylation) affects the ability of the protein to participate in the DNA replication and DNA repair processes.

PCNA has been used as marker for the evaluation of the proliferative state of these malignancies and increased levels of PCNA have been observed in these cancers (i.e., breast, prostate, colon, esophageal, cervical) (Gao *et al.*, 1997; Bleiberg *et al.*, 1993; Kordek *et al.*, 1996; Takasaki *et al.*, 1994; Cordillo *et al.*, 1993; Alexiev, 1996). However there is no correlation between PCNA levels and patient prognosis. The present study demonstrated that the two forms of PCNA were present in a variety of human cancer cells. Prostate cancer, brain cancer, cervical cancer, colon cancer and leukemia cells were shown to contain the acidic form PCNA. The presence of the acidic form of PCNA in these diverse cancers suggests that this form of PCNA may be a fundamental characteristic of malignancy. These results suggest that the acidic form of PCNA has the potential to be a marker for malignancy and may prove to be an indicator of a tumor's proliferative potential. Increased PCNA levels have also been observed in the premalignant colon and prostate lesions compared to nonmalignant tissue (Magdelenat, 1992). This suggests that increased expression of PCNA is an early event in the development of a malignancy. In this report it was shown that transformed cells overexpressing either the c-myc gene or the SV40 T antigen gene contained the acidic form of PCNA. This result indicates that transformation events may contribute to the development of the acidic form of PCNA.

It was proposed that a cell's progression to malignancy is accompanied by the accumulation of multiple genetic mutations created by error-prone DNA replication and a reduction in the efficiency of the DNA repair processes in the cell. Loeb (1998) postulated that PCNA may provide a link between the DNA replication and DNA repair processes of a cell. Therefore an alteration in the function of PCNA could result in a decrease in the fidelity of DNA synthesis. Sekowski *et al.* (submitted) have recently found that the DNA replication

apparatus from malignant breast and leukemia cells was mutagenic, resulting in a decreased replication fidelity. Due to the essential role PCNA plays in both DNA replication and DNA repair, the unique form of PCNA in malignant cells is a potential contributor to the accumulation of genetic mutations and genomic instability.

In addition to the observation and partial charcterization of the PCNA protein in different malignant cells, analysis of a serum sample collected from a breast cancer patient demonstrated that the unique form of PCNA was readily detected in a blood sample. This result suggests that the analysis of serum samples from patients with a variety of human malignancies might also demonstrate the presence of the acidic form of PCNA. We are planning to examine serum samples from individual with in cervical cancer, brain cancer, prostate cancer, and colon cancer in order to determine whether the acidic form of PCNA can be detected. Continued research is needed to determine how the function of the acidic and basic forms of PCNA differ. This information is needed to determine the role of PCNA in the tumorigenic process.

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EXHIBIT 2

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Pamela E. Bechtel, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Robert J. Hickey, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers* (EXHIBIT A), that was included as part of the Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998, and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Linda H. Malkas and Robert J. Hickey are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, and Lawrence Chin are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program.

5. Lori N. Croisetiere was a technician in the laboratory helping Pamela E. Bechtel with her project.

6. Brian J. Long was a post doc who supplied primary breast cells from Dr. Angela Brodie's laboratory.

7. Moshe Talpaz was a division chief at MD Anderson and provided sera from Chronic Myelogenous Leukemia patients under his care and from several of his residents/fellows who served as normal controls for the CML patients.

8. Lawrence Chin provided ovarian cancer tissue.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/3/06

Pamela E. Bechtel

EXHIBIT A

1. *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers*, (Taken from Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998).

Title: An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers.

Authors: Pamela E. Bechtel, Robert J. Hickey, Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, Lawrence Chin and Linda H. Malkas

Key Words: PCNA, tumor marker, post-translational modification, epigenetic change, breast cancer, prostate cancer, malignant glioma, leukemia, cervical cancer, colon cancer

ABSTRACT

The process transforming benign cells into malignant cells involves a complex series of molecular and structural alterations which are poorly understood. One characteristic common to most malignancies is the aberrant proliferation of cells. Cellular proliferation is regulated at a number of points throughout the cell cycle through complex interaction of many proteins. Together these proteins form checkpoints that restrict the ability of cells to progress through the cell cycle. Several critically important checkpoints monitor the cells prior to the entrance into S phase. These checkpoints ultimately signal the DNA synthetic apparatus to begin DNA replication. A multiprotein DNA replication complex, DNA synthesome, has been isolated and characterized from human cells and found to be fully competent to mediate DNA replication *in vitro*. Proliferating cell nuclear antigen (PCNA) was found to be a component of the DNA synthesome. PCNA is an accessory protein for polymerase δ and is involved in DNA repair. A recent study has identified a unique form of PCNA in malignant breast cells that significantly differs in isoelectric point (pI) from the PCNA found in nonmalignant breast cells. The purpose of this study was to determine whether different types of malignant cells contain the altered form of PCNA. To examine this possibility, the DNA synthesome was isolated from malignant prostate cells (LNCaP, PC50), malignant colon cells (KGE90, KYE350, SW48), cervical cancer cells (HeLa), malignant brain cells (T98) and leukemia cells (HL60, CML, AML). The components of the synthesome were resolved by 2 dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA. The results of the analysis demonstrated that the altered form of PCNA was present in all of the malignant cells examined. These findings suggest that the structural alteration of PCNA

may be involved in the development of malignancies due to the critical role PCNA has in the DNA replication and repair processes.

INTRODUCTION

Cancer is the second leading cause of death in the United States (Parker *et al.*, 1997). Lung cancer alone was responsible for more than 160,000 cancer related deaths in 1997, and it is the leading cause of cancer mortality (Parker *et al.*, 1997). Among men, prostate cancer is the second leading cause of cancer related deaths and more than 300,000 new cases were diagnosed in 1997 (Parker *et al.*, 1997). Breast cancer is the second leading cause of cancer related deaths among women and approximately 180,000 new cases of breast cancer were diagnosed in 1997 (Parker *et al.*, 1997). Colon cancer is also a leading cause of cancer deaths in men and women, with 46,000 deaths attributed to this malignancy in 1997 (Parker *et al.*, 1997; Weinberg and Strom, 1995). Leukemias and brain cancers are the most common childhood malignancies with acute lymphocytic leukemia accounting for 80% of childhood cancers (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The origin and clinical manifestations of a malignancy differ for each individual cancer. Breast cancers are usually ductal or lobular in origin and generally affect post menopausal women (Dickson and Lippman, 1992). Prostate and colon cancers generally arise from benign lesions which progress into malignancies. Usually these forms of cancer affect people over the age of fifty (Isaacs, 1997; Meyers and Grizzle, 1997; Parker *et al.*, 1997; Johnson, 1995; Weinberg and Strom, 1995). Many brain tumors arise from astrocytes and are generally refractory to chemotherapy (Kordek *et al.*, 1996; Dalrymple *et al.*, 1994). Cervical cancer has been shown to be caused by human papillomavirus (HPV), and generally occurs in women over the age of thirty five (Villa, 1997; Parker *et al.*, 1997). Leukemias, in contrast to solid tumors, arise in the bone marrow and result in the circulation of immature white cells in the peripheral blood (Kumar *et al.*, 1992). Leukemia can affect people of all ages but children

with leukemias have a significantly increased survival rate compared to adults (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The diagnosis of cancer at an early developmental stage is important in reducing cancer mortality. The use of mammography to detect developing breast cancers has reduced the mortality rate of the disease (Hayes, 1996). Testing for the presence of prostate specific antigen (PSA) has enabled clinicians to diagnose prostate cancer in men early in its onset (Small, 1997; Gao *et al.*, 1997). As a marker, PSA has high sensitivity and specificity and correlates with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). However, a major limitation is the inability to distinguish between benign and malignant prostate lesions by evaluating PSA (Pandha and Waxman, 1995; Magdelenat, 1992). Routine Pap smear screens have been routinely used for the past twenty years and can be beneficial for the early detection and monitoring of cervical cancer among women. Although screening methods exist to detect certain types of malignancy the basic mechanisms for the development of cancer have not been determined.

One commonality of virtually all malignancies is the aberrant proliferation of cells. Aberrant cell proliferation is presumably due to escape from the normal controls which regulate the progression of the cell into specific stages of the cell cycle. Recently, an altered form of the protein proliferating cell nuclear antigen (PCNA) was identified from malignant human breast cells (Bechtel *et al.*, submitted). PCNA is a nuclear protein that is required for DNA synthesis and DNA repair. In this report, PCNA was analyzed from a variety of human malignancies to determine whether the unique form of PCNA was present only in malignant breast cells or whether it was present in other types of malignant cells. Several malignant human cell lines were used: LNCaP and PC50 (prostate adenocarcinoma); KGE90, KYE350 and SW48 (colon adenocarcinoma); T98

(malignant glioma); HeLa (cervical carcinoma); and HL60 (promyelogenous leukemia). PCNA was also analyzed from chronic myelogenous leukemia specimens, acute myelogenous leukemia sample and serum collected from cancer free individuals.

METHODS

Cell culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated from LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and

centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

Analysis of prostate cancer cells in this study demonstrated the presence of the altered form of PCNA. Previous reports indicated that PCNA is strongly expressed in prostate cancers and also has been detected in dysplastic luminal cells of intraepithelial neoplasia, a premalignant prostate lesion (Pandha and Waxman, 1995; Magdelenat, 1992). Gao *et al.* (1997) reported that PCNA levels correlate with the clinical stage and metastatic state of prostate tumors. The researchers suggested that PCNA levels have the potential to be used as a prognostic marker for prostate cancer (Gao *et al.*, 1997).

The colon cancer cell lines examined in this study also contained the altered form of PCNA. Studies have shown that PCNA labeling index is correlated with tritiated thymidine uptake in assessing the proliferative activity of colon tumors (Bleiberg *et al.*, 1993). Neoptolemos *et al.* (1995) demonstrated that PCNA levels used in conjunction with the Duke's classification system (the classification system for colon tumors based on histological examination) improved the prediction of survival for colon cancer patients. Mitchell *et al.* (1997) demonstrated that increased levels of PCNA are found in primary colon tumors and in resulting lung metastases. It has been shown that PCNA levels increase during the progression of benign lesions to malignancy and that increased levels are associated with the recurrence of adenomas (Shpitz *et al.*, 1997; Paspatis *et al.*, 1994; Risio *et al.*, 1993). Risio *et al.* (1993) hypothesized that the increase in the number of PCNA labeled cells is related to hyperproliferation as well as to the neoplastic deregulation of PCNA synthesis in the development of colon cancer.

Malignant gliomas are composed of heterogeneous subpopulations of cells with different cytogenetic compositions making it difficult to identify useful prognostic markers (Dalrymple *et al.*, 1995). Previous studies demonstrated that PCNA labeling index correlates with the histological grade of the brain

malignancy. In the majority of benign astrocytomas, the PCNA labeling index is low (Kordek *et al.*, 1996). Kordek *et al.* (1996) also have hypothesized that malignant gliomas contain two forms of PCNA, one of which does not have a role in DNA synthesis. The results of the present study have indicated that malignant glioma cells contain the altered form of PCNA. The functional role of the altered form of PCNA needs to be examined in greater detail to determine whether it has a role in the development and progression of malignant gliomas.

The role of PCNA in the development and progression of CML is not well characterized. CML is a biphasic disease characterized by an early chronic phase followed by a blast phase (Zaccaria *et al.*, 1995). Takasaki *et al.* (1984b) demonstrated a correlation between the number of leukocytes expressing PCNA and the percent of blast cells in blood during the blast phase of CML. These investigators also identified the presence of non-blast cells which were positive for PCNA in the peripheral blood during the blast phase of CML. This result differs from the observation that the non-blast cells were negative for PCNA in the chronic phase (Takasaki *et al.*, 1984). The PCNA labeling index for CML cells is not significantly different form normal bone marrow cells (Thiele *et al.*, 1993). However, in the chronic myeloid proliferative disorder osteomyelofibrosis, there is a significant increase in the PCNA labeling index (Thiele *et al.*, 1994). Interferon treatment resulted in decreased PCNA labeling. In the present study, the results demonstrated that the leukemia samples examined contain the altered form of PCNA, while samples collected from cancer free individuals did not contain the altered form of PCNA.

Analysis of HeLa cells in the present study revealed that these cervical cancer cells contain the altered form of PCNA. Cardillo *et al.* (1993) found that PCNA levels were increased in all cases of invasive squamous carcinoma, cervical adenoma, cervical carcinoma in situ (CIS) and cervical intraepithelial

neoplasia (CIN) III examined. Smela *et al.* (1984) demonstrated a significant correlation between PCNA index and the severity of cervical dysplasia by determining the PCNA levels in CIN I, CIN II and CIN III lesions. Other studies found that PCNA index was increased in premalignant and malignant cervical lesions compared to nonmalignant tissue (Steinbeck *et al.*, 1995; Raju *et al.*, 1994; Shurbaji, 1993). Kobayski *et al.* (1994) concluded that the increase in PCNA labeling index suggests that there is considerable alteration of biologic activity, including genetic mutations, during the progression of severe cervical dysplasia to CIS.

This study examined several human malignancies for the presence of the acidic form of PCNA. The malignancies examined in this study have few common characteristics or clinical manifestations. Previously, an altered form of PCNA was identified in malignant breast cells. It was demonstrated that the unique form of PCNA was not the result of growth stimulation or genetic mutation (Bechtel *et al.*, submitted). Further research indicated that differential post-translational modification by poly(ADP-ribosylation) was at least partially responsible for the presence of the altered form of the protein (Bechtel *et al.*, submitted). This report identified the acidic form of PCNA in leukemia, prostate, cervical, colon and brain cancers. These data demonstrated a simple molecular alteration in the PCNA polypeptide is a common feature between these diverse types of cancer. These findings suggest that the acidic form of PCNA may have an underlying role in the development of different types of cancer and that these cancers may develop as a consequence of a global alteration in the manner in which specific cellular proteins are post-translational modified.

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FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1

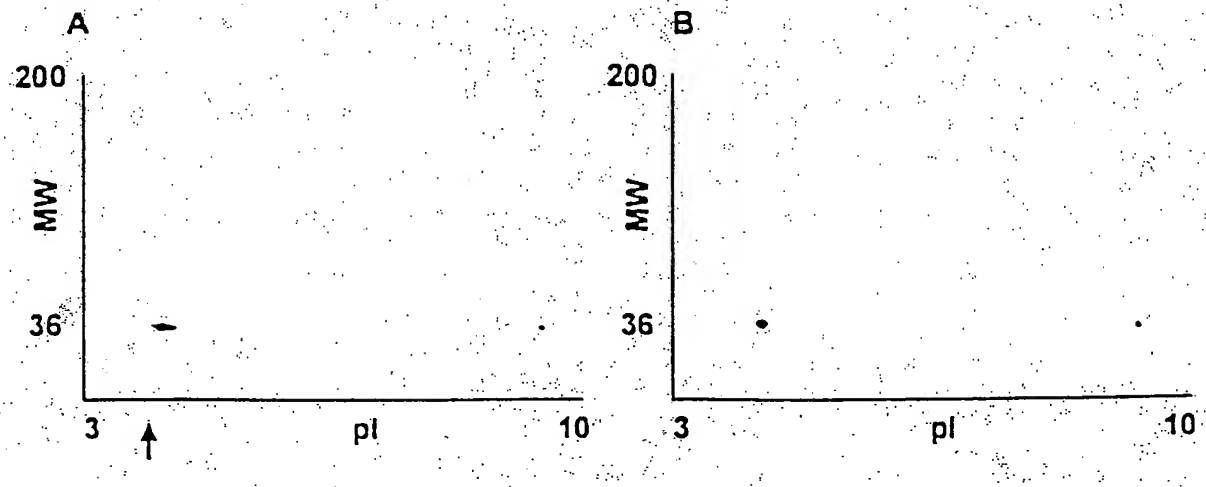


Figure 2

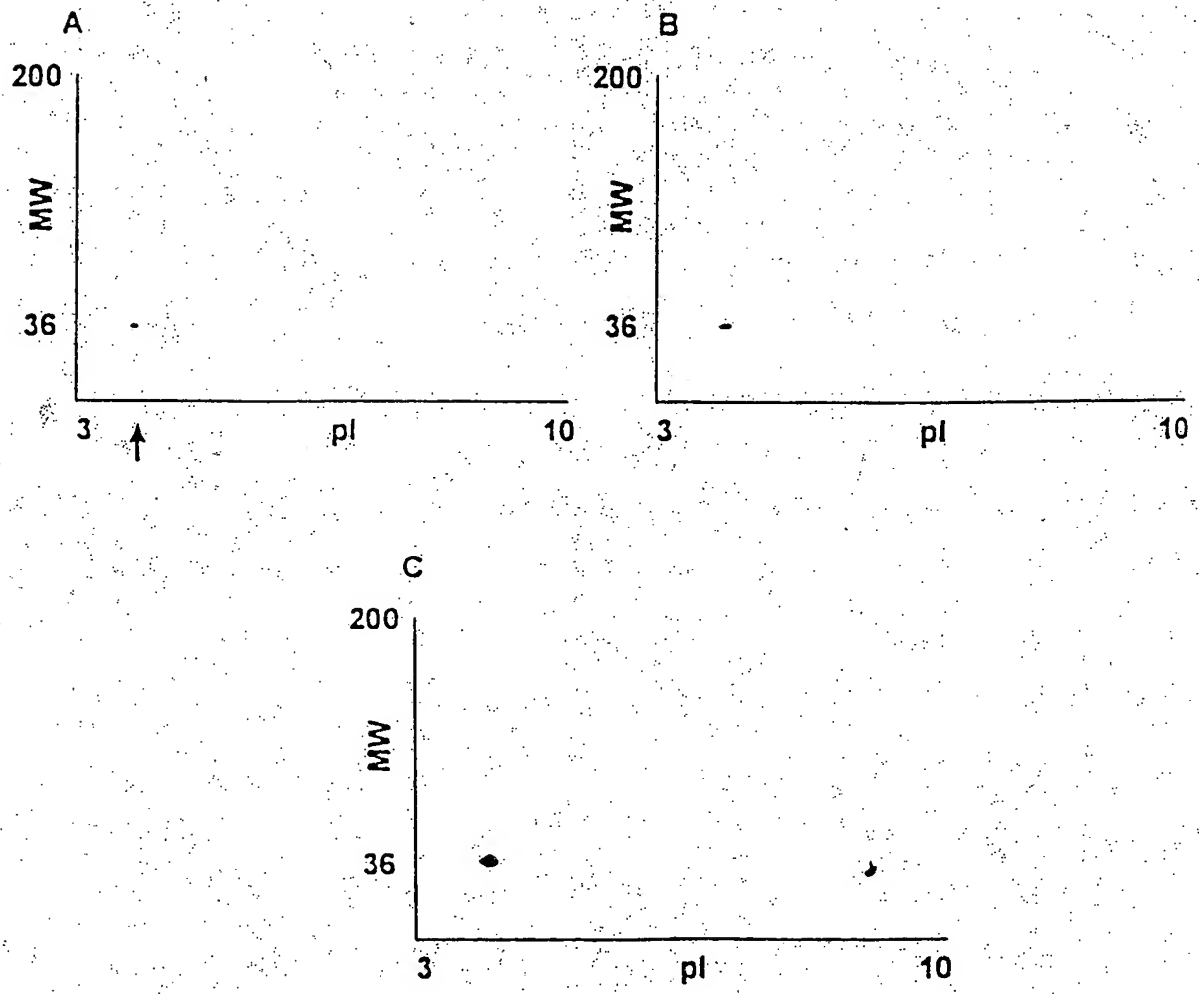


Figure 3

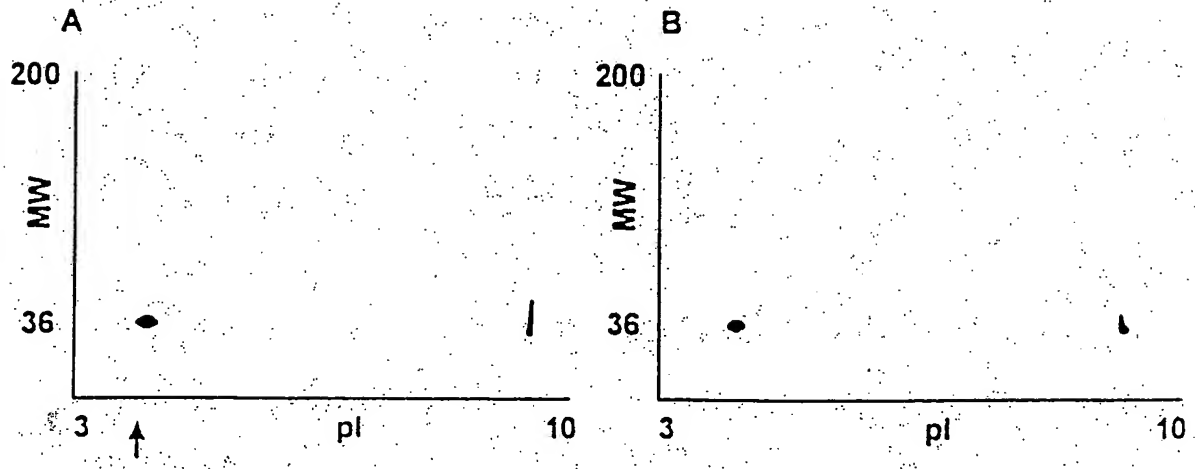
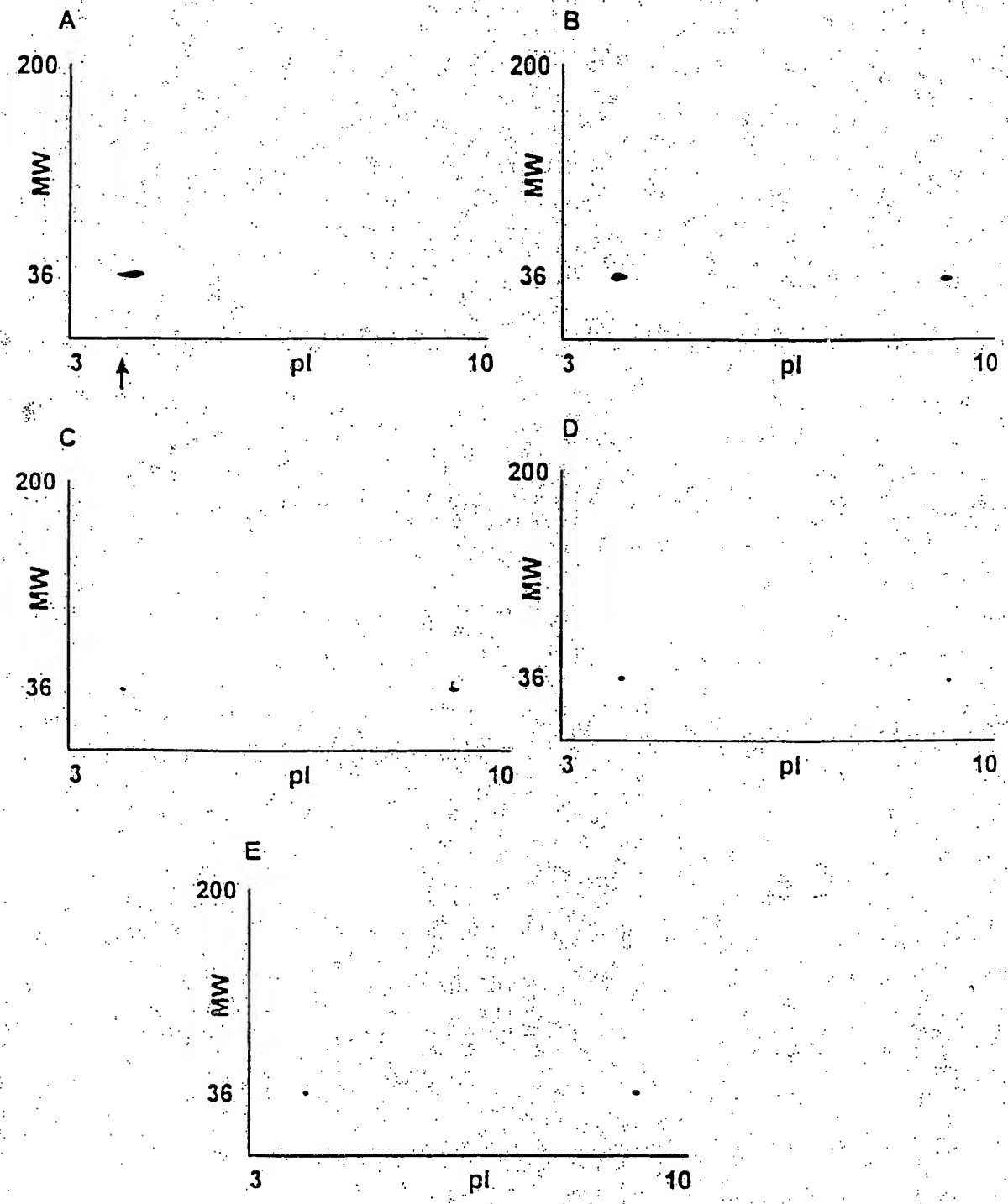


Figure 4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D
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36
3
pI
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EXHIBIT 3

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Robert J. Hickey, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Pamela E. Bechtel, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers* (EXHIBIT A), that was included as part of the Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998, and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Linda H. Malkas and Pamela E. Bechtel are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, and Lawrence Chin are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program.

5. Lori N. Croisetiere was a technician in the laboratory helping Pamela E. Bechtel with her project.

6. Brian J. Long was a post doc who supplied primary breast cells from Dr. Angela Brodie's laboratory.

7. Moshe Talpaz was a division chief at MD Anderson and provided sera from Chronic Myelogenous Leukemia patients under his care and from several of his residents/fellows who served as normal controls for the CML patients.

8. Lawrence Chin provided ovarian cancer tissue.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/3/06

Robert J. Hickey

EXHIBIT A

1. *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers,* (Taken from Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998).

Title: An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers.

Authors: Pamela E. Bechtel, Robert J. Hickey, Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, Lawrence Chin and Linda H. Malkas

Key Words: PCNA, tumor marker, post-translational modification, epigenetic change, breast cancer, prostate cancer, malignant glioma, leukemia, cervical cancer, colon cancer

ABSTRACT

The process transforming benign cells into malignant cells involves a complex series of molecular and structural alterations which are poorly understood. One characteristic common to most malignancies is the aberrant proliferation of cells. Cellular proliferation is regulated at a number of points throughout the cell cycle through complex interaction of many proteins. Together these proteins form checkpoints that restrict the ability of cells to progress through the cell cycle. Several critically important checkpoints monitor the cells prior to the entrance into S phase. These checkpoints ultimately signal the DNA synthetic apparatus to begin DNA replication. A multiprotein DNA replication complex, DNA synthesome, has been isolated and characterized from human cells and found to be fully competent to mediate DNA replication *in vitro*. Proliferating cell nuclear antigen (PCNA) was found to be a component of the DNA synthesome. PCNA is an accessory protein for polymerase δ and is involved in DNA repair. A recent study has identified a unique form of PCNA in malignant breast cells that significantly differs in isoelectric point (pI) from the PCNA found in nonmalignant breast cells. The purpose of this study was to determine whether different types of malignant cells contain the altered form of PCNA. To examine this possibility, the DNA synthesome was isolated from malignant prostate cells (LNCaP, PC50), malignant colon cells (KGE90, KYE350, SW48), cervical cancer cells (HeLa), malignant brain cells (T98) and leukemia cells (HL60, CML, AML). The components of the synthesome were resolved by 2 dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA. The results of the analysis demonstrated that the altered form of PCNA was present in all of the malignant cells examined. These findings suggest that the structural alteration of PCNA

may be involved in the development of malignancies due to the critical role PCNA has in the DNA replication and repair processes.

INTRODUCTION

Cancer is the second leading cause of death in the United States (Parker *et al.*, 1997). Lung cancer alone was responsible for more than 160,000 cancer related deaths in 1997, and it is the leading cause of cancer mortality (Parker *et al.*, 1997). Among men, prostate cancer is the second leading cause of cancer related deaths and more than 300,000 new cases were diagnosed in 1997 (Parker *et al.*, 1997). Breast cancer is the second leading cause of cancer related deaths among women and approximately 180,000 new cases of breast cancer were diagnosed in 1997 (Parker *et al.*, 1997). Colon cancer is also a leading cause of cancer deaths in men and women, with 46,000 deaths attributed to this malignancy in 1997 (Parker *et al.*, 1997; Weinberg and Strom, 1995). Leukemias and brain cancers are the most common childhood malignancies with acute lymphocytic leukemia accounting for 80% of childhood cancers (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The origin and clinical manifestations of a malignancy differ for each individual cancer. Breast cancers are usually ductal or lobular in origin and generally affect post menopausal women (Dickson and Lippman, 1992). Prostate and colon cancers generally arise from benign lesions which progress into malignancies. Usually these forms of cancer affect people over the age of fifty (Isaacs, 1997; Meyers and Grizzle, 1997; Parker *et al.*, 1997; Johnson, 1995; Weinberg and Strom, 1995). Many brain tumors arise from astrocytes and are generally refractory to chemotherapy (Kordek *et al.*, 1996; Dalrymple *et al.*, 1994). Cervical cancer has been shown to be caused by human papillomavirus (HPV), and generally occurs in women over the age of thirty five (Villa, 1997; Parker *et al.*, 1997). Leukemias, in contrast to solid tumors, arise in the bone marrow and result in the circulation of immature white cells in the peripheral blood (Kumar *et al.*, 1992). Leukemia can affect people of all ages but children

with leukemias have a significantly increased survival rate compared to adults (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The diagnosis of cancer at an early developmental stage is important in reducing cancer mortality. The use of mammography to detect developing breast cancers has reduced the mortality rate of the disease (Hayes, 1996). Testing for the presence of prostate specific antigen (PSA) has enabled clinicians to diagnose prostate cancer in men early in its onset (Small, 1997; Gao *et al.*, 1997). As a marker, PSA has high sensitivity and specificity and correlates with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). However, a major limitation is the inability to distinguish between benign and malignant prostate lesions by evaluating PSA (Pandha and Waxman, 1995; Magdelenat, 1992). Routine Pap smear screens have been routinely used for the past twenty years and can be beneficial for the early detection and monitoring of cervical cancer among women. Although screening methods exist to detect certain types of malignancy the basic mechanisms for the development of cancer have not been determined.

One commonality of virtually all malignancies is the aberrant proliferation of cells. Aberrant cell proliferation is presumably due to escape from the normal controls which regulate the progression of the cell into specific stages of the cell cycle. Recently, an altered form of the protein proliferating cell nuclear antigen (PCNA) was identified from malignant human breast cells (Bechtel *et al.*, submitted). PCNA is a nuclear protein that is required for DNA synthesis and DNA repair. In this report, PCNA was analyzed from a variety of human malignancies to determine whether the unique form of PCNA was present only in malignant breast cells or whether it was present in other types of malignant cells. Several malignant human cell lines were used: LNCaP and PC50 (prostate adenocarcinoma); KGE90, KYE350 and SW48 (colon adenocarcinoma); T98

(malignant glioma); HeLa (cervical carcinoma); and HL60 (promyelogenous leukemia). PCNA was also analyzed from chronic myelogenous leukemia specimens, acute myelogenous leukemia sample and serum collected from cancer free individuals.

METHODS

Cell culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated from LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and

centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

Analysis of prostate cancer cells in this study demonstrated the presence of the altered form of PCNA. Previous reports indicated that PCNA is strongly expressed in prostate cancers and also has been detected in dysplastic luminal cells of intraepithelial neoplasia, a premalignant prostate lesion (Pandha and Waxman, 1995; Magdelenat, 1992). Gao *et al.* (1997) reported that PCNA levels correlate with the clinical stage and metastatic state of prostate tumors. The researchers suggested that PCNA levels have the potential to be used as a prognostic marker for prostate cancer (Gao *et al.*, 1997).

The colon cancer cell lines examined in this study also contained the altered form of PCNA. Studies have shown that PCNA labeling index is correlated with tritiated thymidine uptake in assessing the proliferative activity of colon tumors (Bleiberg *et al.*, 1993). Neoptolemos *et al.* (1995) demonstrated that PCNA levels used in conjunction with the Duke's classification system (the classification system for colon tumors based on histological examination) improved the prediction of survival for colon cancer patients. Mitchell *et al.* (1997) demonstrated that increased levels of PCNA are found in primary colon tumors and in resulting lung metastases. It has been shown that PCNA levels increase during the progression of benign lesions to malignancy and that increased levels are associated with the recurrence of adenomas (Shpitz *et al.*, 1997; Paspatis *et al.*, 1994; Risio *et al.*, 1993). Risio *et al.* (1993) hypothesized that the increase in the number of PCNA labeled cells is related to hyperproliferation as well as to the neoplastic deregulation of PCNA synthesis in the development of colon cancer.

Malignant gliomas are composed of heterogeneous subpopulations of cells with different cytogenetic compositions making it difficult to identify useful prognostic markers (Dalrymple *et al.*, 1995). Previous studies demonstrated that PCNA labeling index correlates with the histological grade of the brain

malignancy. In the majority of benign astrocytomas, the PCNA labeling index is low (Kordek *et al.*, 1996). Kordek *et al.* (1996) also have hypothesized that malignant gliomas contain two forms of PCNA, one of which does not have a role in DNA synthesis. The results of the present study have indicated that malignant glioma cells contain the altered form of PCNA. The functional role of the altered form of PCNA needs to be examined in greater detail to determine whether it has a role in the development and progression of malignant gliomas.

The role of PCNA in the development and progression of CML is not well characterized. CML is a biphasic disease characterized by an early chronic phase followed by a blast phase (Zaccaria *et al.*, 1995). Takasaki *et al.* (1984b) demonstrated a correlation between the number of leukocytes expressing PCNA and the percent of blast cells in blood during the blast phase of CML. These investigators also identified the presence of non-blast cells which were positive for PCNA in the peripheral blood during the blast phase of CML. This result differs from the observation that the non-blast cells were negative for PCNA in the chronic phase (Takasaki *et al.*, 1984). The PCNA labeling index for CML cells is not significantly different form normal bone marrow cells (Thiele *et al.*, 1993). However, in the chronic myeloid proliferative disorder osteomyelofibrosis, there is a significant increase in the PCNA labeling index (Thiele *et al.*, 1994). Interferon treatment resulted in decreased PCNA labeling. In the present study, the results demonstrated that the leukemia samples examined contain the altered form of PCNA, while samples collected from cancer free individuals did not contain the altered form of PCNA.

Analysis of HeLa cells in the present study revealed that these cervical cancer cells contain the altered form of PCNA. Cardillo *et al.* (1993) found that PCNA levels were increased in all cases of invasive squamous carcinoma, cervical adenoma, cervical carcinoma in situ (CIS) and cervical intraepithelial

neoplasia (CIN) III examined. Smela *et al.* (1984) demonstrated a significant correlation between PCNA index and the severity of cervical dysplasia by determining the PCNA levels in CIN I, CIN II and CIN III lesions. Other studies found that PCNA index was increased in premalignant and malignant cervical lesions compared to nonmalignant tissue (Steinbeck *et al.*, 1995; Raju *et al.*, 1994; Shurbaji, 1993). Kobayski *et al.* (1994) concluded that the increase in PCNA labeling index suggests that there is considerable alteration of biologic activity, including genetic mutations, during the progression of severe cervical dysplasia to CIS.

This study examined several human malignancies for the presence of the acidic form of PCNA. The malignancies examined in this study have few common characteristics or clinical manifestations. Previously, an altered form of PCNA was identified in malignant breast cells. It was demonstrated that the unique form of PCNA was not the result of growth stimulation or genetic mutation (Bechtel *et al.*, submitted). Further research indicated that differential post-translational modification by poly(ADP-ribosylation) was at least partially responsible for the presence of the altered form of the protein (Bechtel *et al.*, submitted). This report identified the acidic form of PCNA in leukemia, prostate, cervical, colon and brain cancers. These data demonstrated a simple molecular alteration in the PCNA polypeptide is a common feature between these diverse types of cancer. These findings suggest that the acidic form of PCNA may have an underlying role in the development of different types of cancer and that these cancers may develop as a consequence of a global alteration in the manner in which specific cellular proteins are post-translational modified.

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FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1

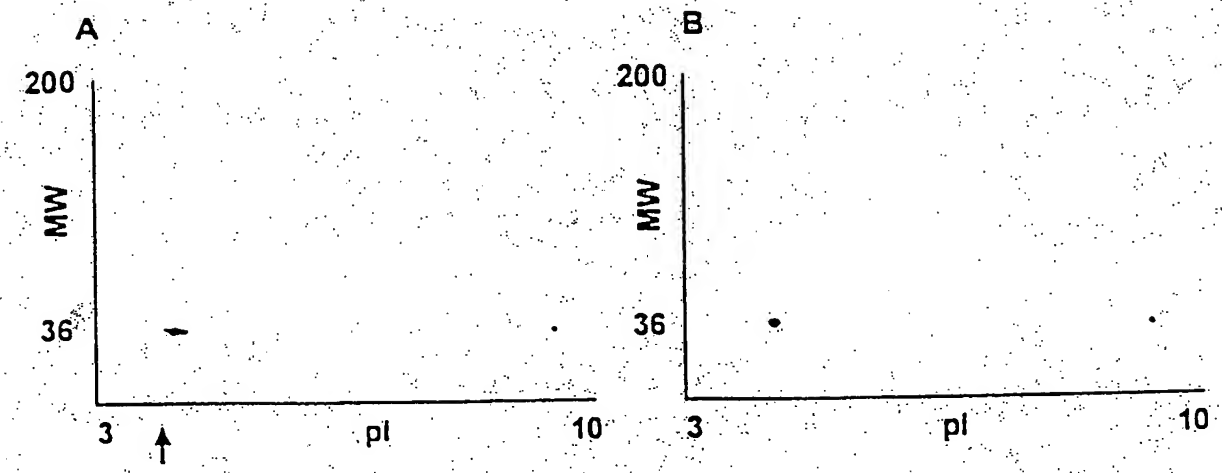


Figure 2

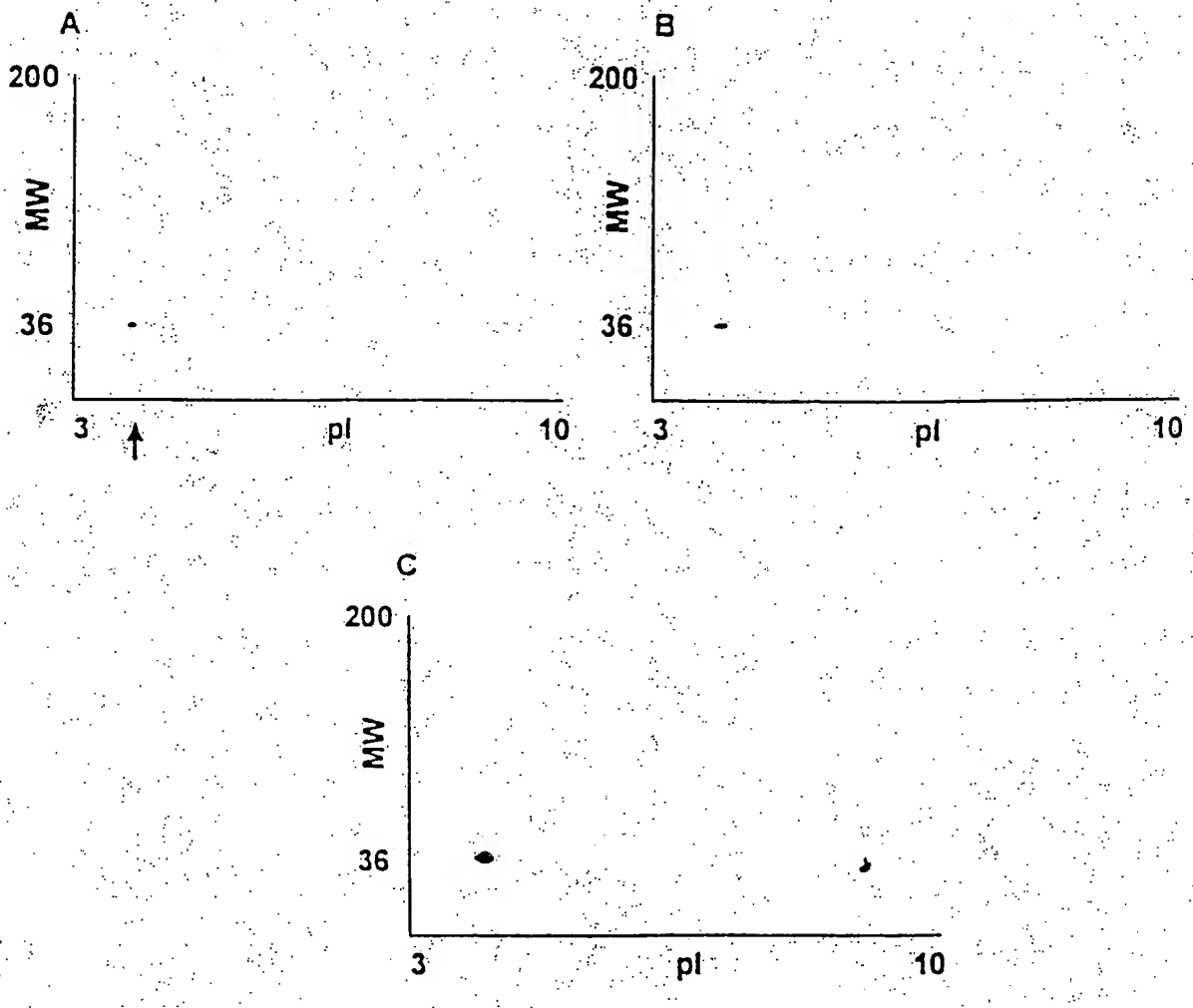


Figure 3

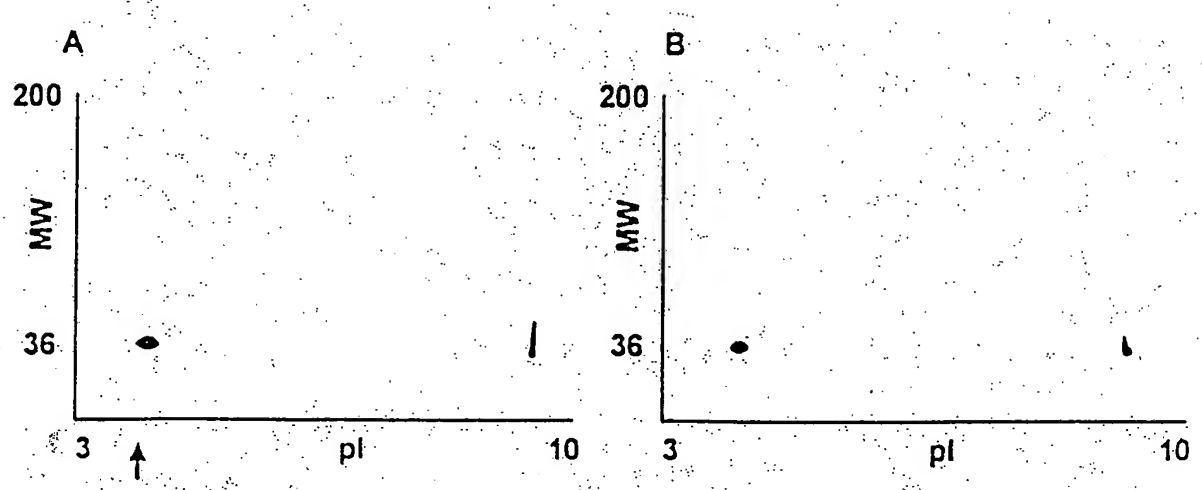
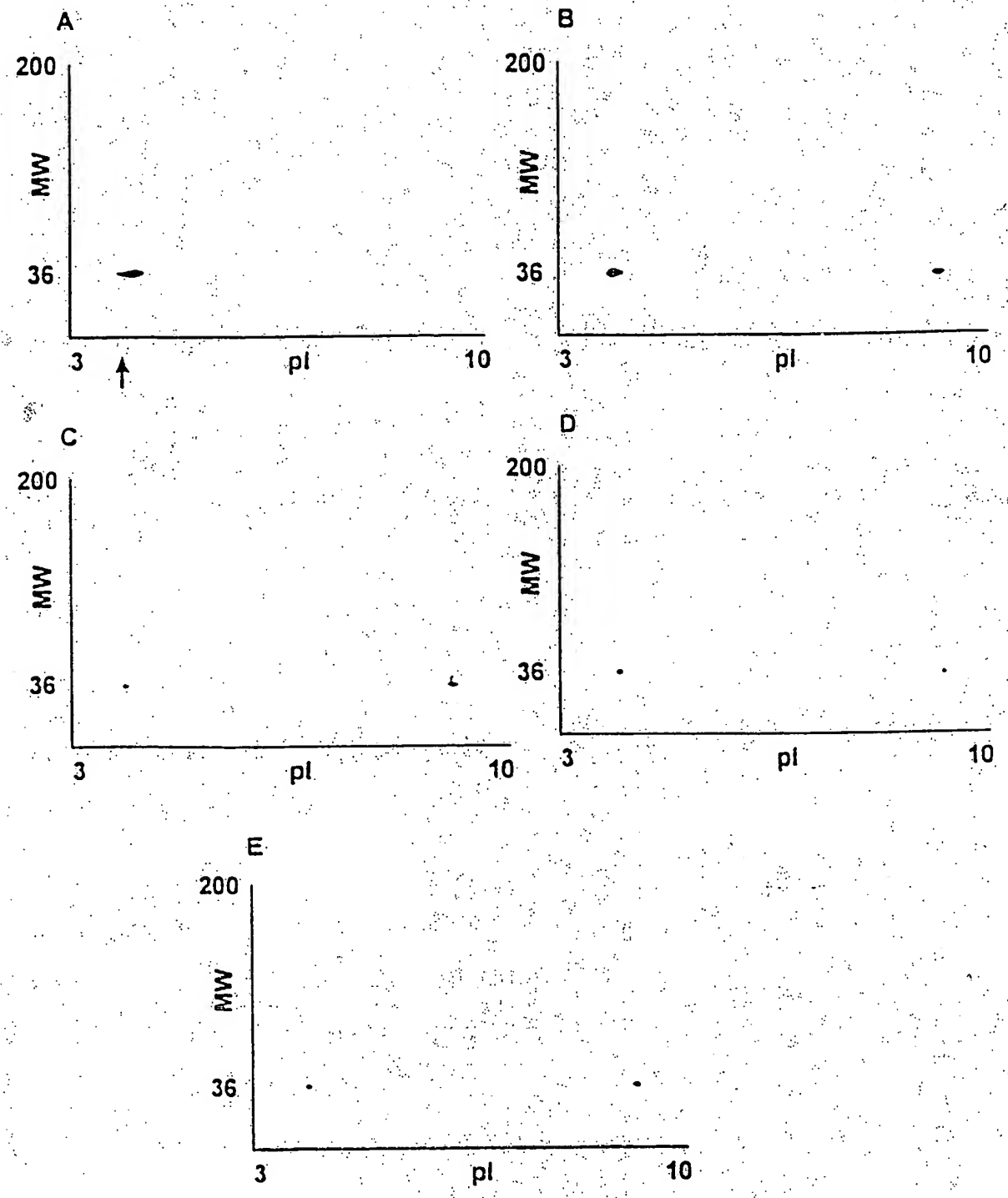


Figure 4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3
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EXHIBIT 4

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Linda H. Malkas, declare as follows:

1. I am a co-inventor, along with Robert J. Hickey, Pamela E. Bechtel, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers* (EXHIBIT A), that was included as part of the Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998, and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey and Pamela E. Bechtel are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, and Lawrence Chin are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program.

5. Lori N. Croisetiere was a technician in the laboratory helping Pamela E. Bechtel with her project.

6. Brian J. Long was a post doc who supplied primary breast cells from Dr. Angela Brodie's laboratory.

7. Moshe Talpaz was a division chief at MD Anderson and provided sera from Chronic Myelogenous Leukemia patients under his care and from several of his residents/fellows who served as normal controls for the CML patients.

8. Lawrence Chin provided ovarian cancer tissue.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/8/06

Linda H. Malkas

EXHIBIT A

1. *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers*, (Taken from Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998).

Title: An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers.

Authors: Pamela E. Bechtel, Robert J. Hickey, Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, Lawrence Chin and Linda H. Malkas

Key Words: PCNA, tumor marker, post-translational modification, epigenetic change, breast cancer, prostate cancer, malignant glioma, leukemia, cervical cancer, colon cancer

ABSTRACT

The process transforming benign cells into malignant cells involves a complex series of molecular and structural alterations which are poorly understood. One characteristic common to most malignancies is the aberrant proliferation of cells. Cellular proliferation is regulated at a number of points throughout the cell cycle through complex interaction of many proteins. Together these proteins form checkpoints that restrict the ability of cells to progress through the cell cycle. Several critically important checkpoints monitor the cells prior to the entrance into S phase. These checkpoints ultimately signal the DNA synthetic apparatus to begin DNA replication. A multiprotein DNA replication complex, DNA synthesome, has been isolated and characterized from human cells and found to be fully competent to mediate DNA replication *in vitro*. Proliferating cell nuclear antigen (PCNA) was found to be a component of the DNA synthesome. PCNA is an accessory protein for polymerase δ and is involved in DNA repair. A recent study has identified a unique form of PCNA in malignant breast cells that significantly differs in isoelectric point (pI) from the PCNA found in nonmalignant breast cells. The purpose of this study was to determine whether different types of malignant cells contain the altered form of PCNA. To examine this possibility, the DNA synthesome was isolated from malignant prostate cells (LNCaP, PC50), malignant colon cells (KGE90, KYE350, SW48), cervical cancer cells (HeLa), malignant brain cells (T98) and leukemia cells (HL60, CML, AML). The components of the synthesome were resolved by 2 dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA. The results of the analysis demonstrated that the altered form of PCNA was present in all of the malignant cells examined. These findings suggest that the structural alteration of PCNA

may be involved in the development of malignancies due to the critical role PCNA has in the DNA replication and repair processes.

INTRODUCTION

Cancer is the second leading cause of death in the United States (Parker *et al.*, 1997). Lung cancer alone was responsible for more than 160,000 cancer related deaths in 1997, and it is the leading cause of cancer mortality (Parker *et al.*, 1997). Among men, prostate cancer is the second leading cause of cancer related deaths and more than 300,000 new cases were diagnosed in 1997 (Parker *et al.*, 1997). Breast cancer is the second leading cause of cancer related deaths among women and approximately 180,000 new cases of breast cancer were diagnosed in 1997 (Parker *et al.*, 1997). Colon cancer is also a leading cause of cancer deaths in men and women, with 46,000 deaths attributed to this malignancy in 1997 (Parker *et al.*, 1997; Weinberg and Strom, 1995). Leukemias and brain cancers are the most common childhood malignancies with acute lymphocytic leukemia accounting for 80% of childhood cancers (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The origin and clinical manifestations of a malignancy differ for each individual cancer. Breast cancers are usually ductal or lobular in origin and generally affect post menopausal women (Dickson and Lippman, 1992). Prostate and colon cancers generally arise from benign lesions which progress into malignancies. Usually these forms of cancer affect people over the age of fifty (Isaacs, 1997; Meyers and Grizzle, 1997; Parker *et al.*, 1997; Johnson, 1995; Weinberg and Strom, 1995). Many brain tumors arise from astrocytes and are generally refractory to chemotherapy (Kordek *et al.*, 1996; Dalrymple *et al.*, 1994). Cervical cancer has been shown to be caused by human papillomavirus (HPV), and generally occurs in women over the age of thirty five (Villa, 1997; Parker *et al.*, 1997). Leukemias, in contrast to solid tumors, arise in the bone marrow and result in the circulation of immature white cells in the peripheral blood (Kumar *et al.*, 1992). Leukemia can affect people of all ages but children

with leukemias have a significantly increased survival rate compared to adults (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The diagnosis of cancer at an early developmental stage is important in reducing cancer mortality. The use of mammography to detect developing breast cancers has reduced the mortality rate of the disease (Hayes, 1996). Testing for the presence of prostate specific antigen (PSA) has enabled clinicians to diagnose prostate cancer in men early in its onset (Small, 1997; Gao *et al.*, 1997). As a marker, PSA has high sensitivity and specificity and correlates with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). However, a major limitation is the inability to distinguish between benign and malignant prostate lesions by evaluating PSA (Pandha and Waxman, 1995; Magdelenat, 1992). Routine Pap smear screens have been routinely used for the past twenty years and can be beneficial for the early detection and monitoring of cervical cancer among women. Although screening methods exist to detect certain types of malignancy the basic mechanisms for the development of cancer have not been determined.

One commonality of virtually all malignancies is the aberrant proliferation of cells. Aberrant cell proliferation is presumably due to escape from the normal controls which regulate the progression of the cell into specific stages of the cell cycle. Recently, an altered form of the protein proliferating cell nuclear antigen (PCNA) was identified from malignant human breast cells (Bechtel *et al.*, submitted). PCNA is a nuclear protein that is required for DNA synthesis and DNA repair. In this report, PCNA was analyzed from a variety of human malignancies to determine whether the unique form of PCNA was present only in malignant breast cells or whether it was present in other types of malignant cells. Several malignant human cell lines were used: LNCaP and PC50 (prostate adenocarcinoma); KGE90, KYE350 and SW48 (colon adenocarcinoma); T98

(malignant glioma); HeLa (cervical carcinoma); and HL60 (promyelogenous leukemia). PCNA was also analyzed from chronic myelogenous leukemia specimens, acute myelogenous leukemia sample and serum collected from cancer free individuals.

METHODS

Cell culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated from LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and

centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

Analysis of prostate cancer cells in this study demonstrated the presence of the altered form of PCNA. Previous reports indicated that PCNA is strongly expressed in prostate cancers and also has been detected in dysplastic luminal cells of intraepithelial neoplasia, a premalignant prostate lesion (Pandha and Waxman, 1995; Magdelenat, 1992). Gao *et al.* (1997) reported that PCNA levels correlate with the clinical stage and metastatic state of prostate tumors. The researchers suggested that PCNA levels have the potential to be used as a prognostic marker for prostate cancer (Gao *et al.*, 1997).

The colon cancer cell lines examined in this study also contained the altered form of PCNA. Studies have shown that PCNA labeling index is correlated with tritiated thymidine uptake in assessing the proliferative activity of colon tumors (Bleiberg *et al.*, 1993). Neoptolemos *et al.* (1995) demonstrated that PCNA levels used in conjunction with the Duke's classification system (the classification system for colon tumors based on histological examination) improved the prediction of survival for colon cancer patients. Mitchell *et al.* (1997) demonstrated that increased levels of PCNA are found in primary colon tumors and in resulting lung metastases. It has been shown that PCNA levels increase during the progression of benign lesions to malignancy and that increased levels are associated with the recurrence of adenomas (Shpitz *et al.*, 1997; Paspatis *et al.*, 1994; Risio *et al.*, 1993). Risio *et al.* (1993) hypothesized that the increase in the number of PCNA labeled cells is related to hyperproliferation as well as to the neoplastic deregulation of PCNA synthesis in the development of colon cancer.

Malignant gliomas are composed of heterogeneous subpopulations of cells with different cytogenetic compositions making it difficult to identify useful prognostic markers (Dalrymple *et al.*, 1995). Previous studies demonstrated that PCNA labeling index correlates with the histological grade of the brain

malignancy. In the majority of benign astrocytomas, the PCNA labeling index is low (Kordek *et al.*, 1996). Kordek *et al.* (1996) also have hypothesized that malignant gliomas contain two forms of PCNA, one of which does not have a role in DNA synthesis. The results of the present study have indicated that malignant glioma cells contain the altered form of PCNA. The functional role of the altered form of PCNA needs to be examined in greater detail to determine whether it has a role in the development and progression of malignant gliomas.

The role of PCNA in the development and progression of CML is not well characterized. CML is a biphasic disease characterized by an early chronic phase followed by a blast phase (Zaccaria *et al.*, 1995). Takasaki *et al.* (1984b) demonstrated a correlation between the number of leukocytes expressing PCNA and the percent of blast cells in blood during the blast phase of CML. These investigators also identified the presence of non-blast cells which were positive for PCNA in the peripheral blood during the blast phase of CML. This result differs from the observation that the non-blast cells were negative for PCNA in the chronic phase (Takasaki *et al.*, 1984). The PCNA labeling index for CML cells is not significantly different form normal bone marrow cells (Thiele *et al.*, 1993). However, in the chronic myeloid proliferative disorder osteomyelofibrosis, there is a significant increase in the PCNA labeling index (Thiele *et al.*, 1994). Interferon treatment resulted in decreased PCNA labeling. In the present study, the results demonstrated that the leukemia samples examined contain the altered form of PCNA, while samples collected from cancer free individuals did not contain the altered form of PCNA.

Analysis of HeLa cells in the present study revealed that these cervical cancer cells contain the altered form of PCNA. Cardillo *et al.* (1993) found that PCNA levels were increased in all cases of invasive squamous carcinoma, cervical adenoma, cervical carcinoma in situ (CIS) and cervical intraepithelial

neoplasia (CIN) III examined. Smela *et al.* (1984) demonstrated a significant correlation between PCNA index and the severity of cervical dysplasia by determining the PCNA levels in CIN I, CIN II and CIN III lesions. Other studies found that PCNA index was increased in premalignant and malignant cervical lesions compared to nonmalignant tissue (Steinbeck *et al.*, 1995; Raju *et al.*, 1994; Shurbaji, 1993). Kobayski *et al.* (1994) concluded that the increase in PCNA labeling index suggests that there is considerable alteration of biologic activity, including genetic mutations, during the progression of severe cervical dysplasia to CIS.

This study examined several human malignancies for the presence of the acidic form of PCNA. The malignancies examined in this study have few common characteristics or clinical manifestations. Previously, an altered form of PCNA was identified in malignant breast cells. It was demonstrated that the unique form of PCNA was not the result of growth stimulation or genetic mutation (Bechtel *et al.*, submitted). Further research indicated that differential post-translational modification by poly(ADP-ribosylation) was at least partially responsible for the presence of the altered form of the protein (Bechtel *et al.*, submitted). This report identified the acidic form of PCNA in leukemia, prostate, cervical, colon and brain cancers. These data demonstrated a simple molecular alteration in the PCNA polypeptide is a common feature between these diverse types of cancer. These findings suggest that the acidic form of PCNA may have an underlying role in the development of different types of cancer and that these cancers may develop as a consequence of a global alteration in the manner in which specific cellular proteins are post-translational modified.

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FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1

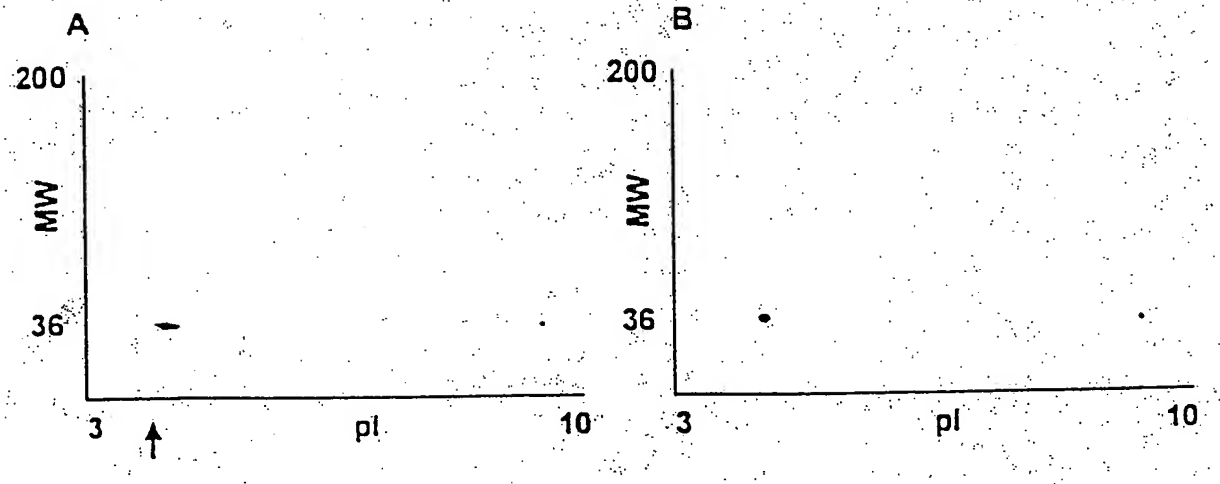

Figure 2

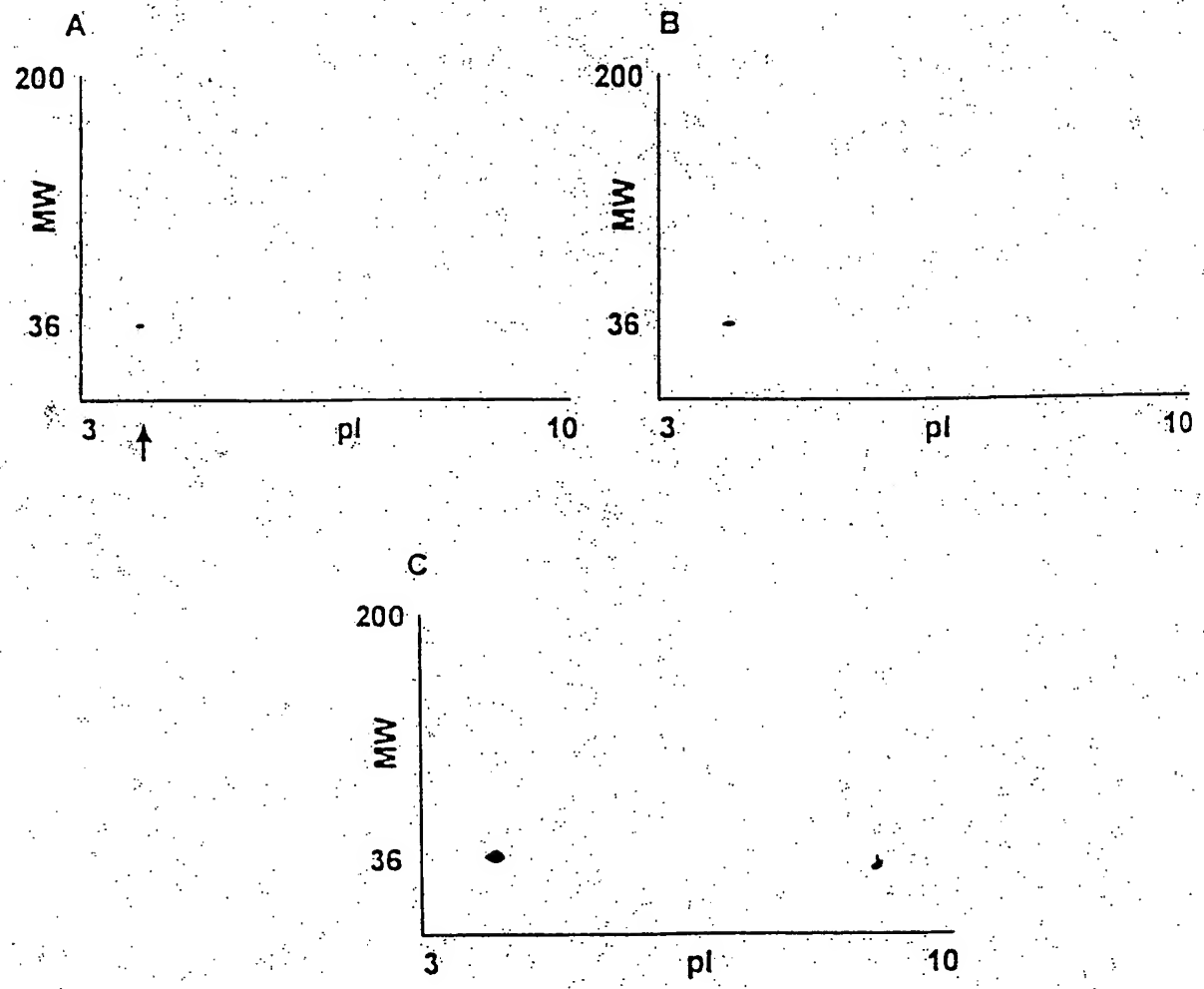

Figure 3

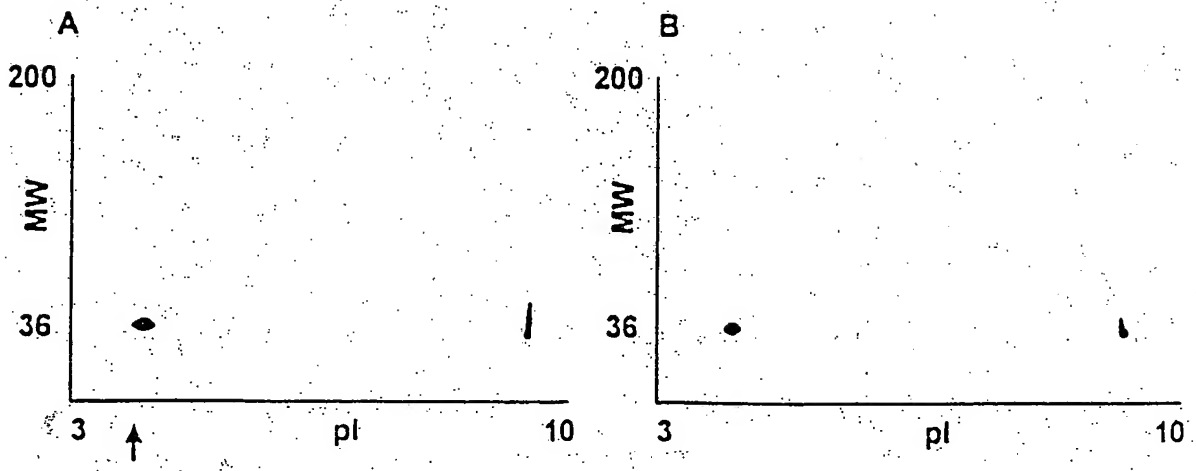

Figure 4

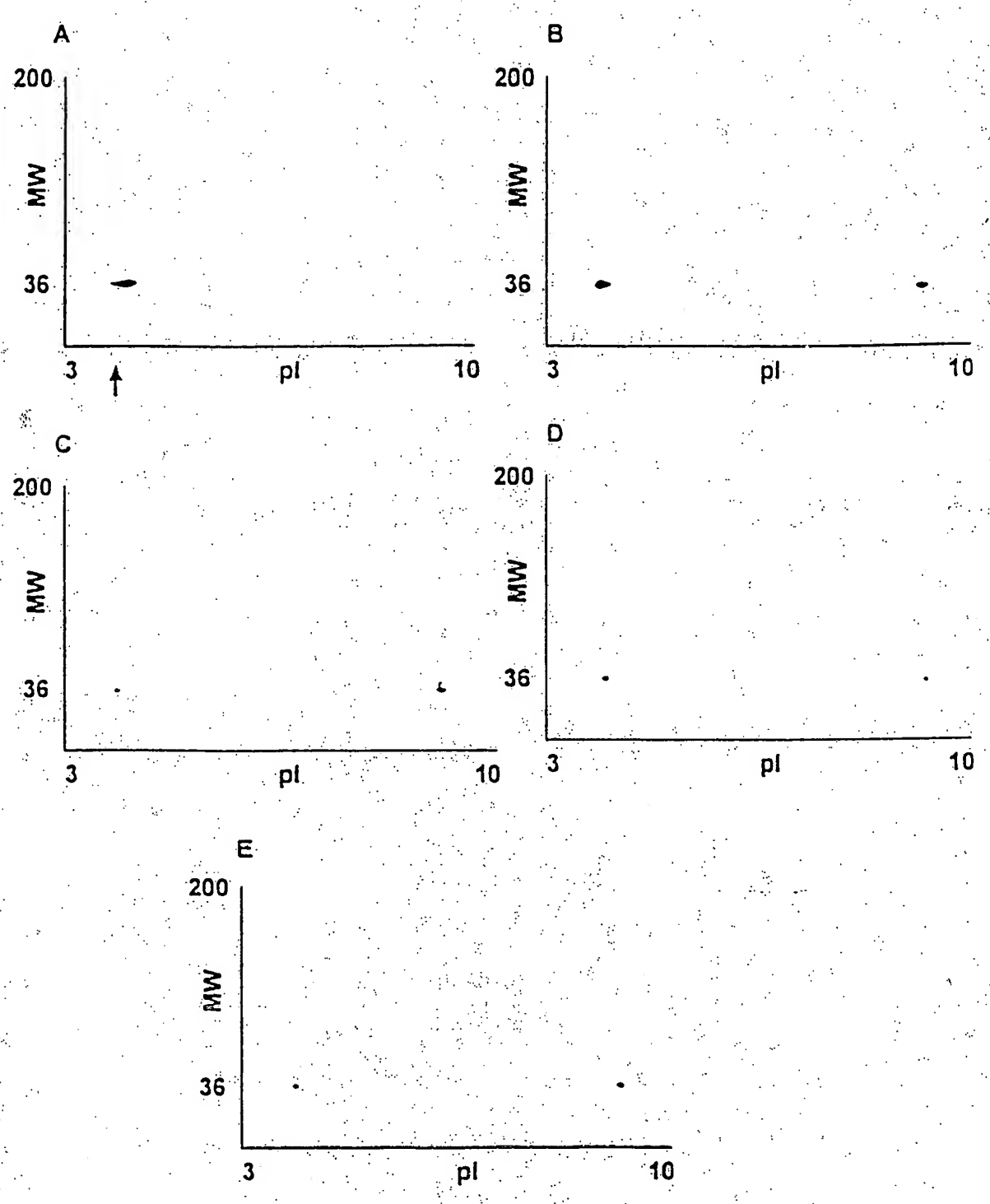
A
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EXHIBIT 5

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Robert J. Hickey, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Pamela E. Bechtel, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Linda H. Malkas, Lauren Schnaper, Derek J. Hoelz, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/3/06

Robert J. Hickey

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

#2507 Detection of the Cancer Specific Form of PCNA by Elisa Assay. D. Tomic, D. J. Hoelz, P. Wills, R. J. Hickey, L. Schnaper, C. Lankford, and L. H. Malkas. *Greater Baltimore Medical Center, Towson, MD, and University of Maryland, Baltimore, MD.*

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which is required for DNA replication (processivity factor of DNA polymerase δ) and DNA repair. Previously, using 2D-IEF-PAGE analyses, our laboratory discovered that malignant breast cells express a unique, acidic form of PCNA protein which can clearly be distinguished from the basic form of this protein found in non-malignant cells. Our research suggests that the acidic form of PCNA is, most likely, the result of a post translational modification. This finding is important because this unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of this malignancy. Therefore, the purpose of this study was to develop an ELISA test, which can distinguish the malignant form of PCNA from the non-malignant form. We tested the hypothesis that xeroderma pigmentosum (XP)G protein, a structure-specific repair endonuclease similar to FEN1, and used in the nucleotide excision repair pathway is capable of distinguishing two forms of PCNA through binding affinities. To test this hypothesis, the protein isolated from the non-malignant breast cell line (MCF10A) and the breast cancer cell line (MCF-7) were used to measure the binding affinity of XPG to the acidic and basic form of PCNA in a modified ELISA assay. Standard curves, representing the correlation between absorbance and the abundance of the malignant and non-malignant form of PCNA were prepared and compared to each other. Serial dilutions of PCNA were tested in duplicate and the mean value of absorbance was calculated and used for comparison. Our results indicate that XPG protein has a different binding affinity for the malignant and non-malignant forms of PCNA. These results are the first to demonstrate that these two forms of PCNA can be distinguished by an ELISA assay that can be used clinically for the early detection of breast cancer.

#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
occupancy show similar binding
ylguanine. We conclude, therefore, that while zinc is neither essential for DN repair by hAGT nor required for maintaining a functional fold of the protein, t presence of the transition metal ion bound within the polypeptide structu confers a mechanistic enhancement to repair activity which does not result fro an increase in substrate binding affinity. Zinc may also provide some measure structural stability to hAGT.

#2509 Molecular Alterations in the Transcription-Coupled Nucleotide Excision Repair Gene, CS-B/ERCC6, in Human Malignant Gliomas. Franc Ali-Osman, Kurt Jaeckle, Thomas Connor, Gamil Antoun, and Lixin Zhan *U.T. M.D. Anderson Cancer Center, Houston, TX.*

The CS-B/ERCC6 gene encodes a complementation factor required for ef cient transcription-coupled nucleotide excision repair (TC-NER), a major DN repair pathway by which a variety of lesions are removed from the cellul genome. In this study, we examined 39 primary human malignant glioma spec mens and their matched normal tissues, as well as, 11 early passage glioma ce lines, for molecular alterations (deletions and mutations) in the CS-B gene. Th results were correlated with the histological grade of the tumors. The result showed, overall, CS-B gene deletions to increase with increasing glioma grad and exon II to be most frequently deleted exon. Frequencies of exon II deletion were 12.5%, 30.8% and 66.7%, in astrocytomas, anaplastic astrocytomas an glioblastoma multiformes, respectively. Mutation analysis performed by SSC analysis of exon II and confirmed by nucleotide sequencing, showed a lowe frequency of mutations in exon II of the CS-B gene in the tumors, with only 12.5% of the glioma, all anaplastic astrocytoma or glioblastoma multiforme, to harbo any mutations. The mutations were varied and comprised of nucleotide trans tions of CAC(H)→TAC(Y) in codon 13 and CAA(C)→CGG(H) in codon 15, an transversions of TCT(S)→TAT(Y) and CAG(Q)→CAC(H) in codons 57 and 7 respectively. These data suggest that defective TC-NER, resulting from genet abnormalities in the CS-B gene, particularly, in exons 2 and 5, may contribute t malignant progression in gliomas.

#2510 Y-Box Binding Protein-1 Binds Preferentially to Single-Stranded Nucleic Acid and Exhibits 3' - 5' Exonuclease Activity. Hiroto Izumi, Toshihir Imamura, Gunji Nagatani, Tomoko Ise, Tadashi Murakami, Hidetaka Uramoto, Takayuki Torigoe, Hiroshi Ishiguchi, Yoichiro Yoshida, Minoru Nomoto, and Kim toshi Kohno. *Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan, and University of Occupational and Environmental Health, Fukuoka, Japan.*

We previously have shown that YB-1 (Y-box binding protein-1) binds prefe entially to cisplatin-modified Y-box sequences. Based on structural and biochem ical data, we predicted that this protein binds single-stranded nucleic acids. In th present study we confirmed the prediction and also discovered some unexpecte functional features of Y-box binding protein-1. We found that the cold-shoc domain of the protein is necessary but not sufficient for double-stranded DN binding while the C-tail domain interacts with both single-stranded DNA and RN independently of the cold-shock domain. In an in vitro translation systems, th C-tail domain of the protein inhibited translation but the cold-shock domain di not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonu cleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletio analysis mapped the C-tail domain of the protein as the region of homodimer ization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of th protein. The region between residues 51 and 205 of its 324-amino acid extent i required for full exonuclease activity. Our findings suggest that Y-box bindin protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

3'-5' exonucleases are proteins that ensure the accuracy of DNA replication b catalyzing the removal of mispaired nucleotides from the 3'-termini of nascen DNA. Base mutations arising through nucleotide misinsertions are considered t be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α lacks an intrinsic proofreading exonuclease, although it has been suggested tha an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of eithe primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripher blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exo nuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown tha exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides

EXHIBIT 6

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Linda H. Malkas, declare as follows:

1. I am a co-inventor, along with Robert J. Hickey, Pamela E. Bechtel, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Lauren Schnaper, Derek J. Hoelz, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/9/06

Linda H. Malkas

EXHIBIT A

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O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

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#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
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#2509 Molecular Alterations in the Transcription-Coupled Nucleotide Excision Repair Gene, CS-B/ERCC6, in Human Malignant Gliomas. Franc Ali-Osman, Kurt Jaeckle, Thomas Connor, Gamil Antoun, and Lixin Zhan *U.T. M.D. Anderson Cancer Center, Houston, TX.*

The CS-B/ERCC6 gene encodes a complementation factor required for effi cient transcription-coupled nucleotide excision repair (TC-NER), a major DN repair pathway by which a variety of lesions are removed from the cellula genome. In this study, we examined 39 primary human malignant glioma spec mens and their matched normal tissues, as well as, 11 early passage glioma ce lines, for molecular alterations (deletions and mutations) in the CS-B gene. Th results were correlated with the histological grade of the tumors. The result showed, overall, CS-B gene deletions to increase with increasing glioma grad and exon II to be most frequently deleted exon. Frequencies of exon II deletion were 12.5%, 30.8% and 66.7%, in astrocytomas, anaplastic astrocytomas an glioblastoma multiformes, respectively. Mutation analysis performed by SSC analysis of exon II and confirmed by nucleotide sequencing, showed a lowe frequency of mutations in exon II of the CS-B gene in the tumors, with only 12.5% of the glioma, all anaplastic astrocytoma or glioblastoma multiforme, to harbo any mutations. The mutations were varied and comprised of nucleotide trans tions of CAC(H)→TAC(Y) in codon 13 and CAA(C)→CGG(H) in codon 15, an transversions of TCT(S)→TAT(Y) and CAG(Q)→CAC(H) in codons 57 and 7 respectively. These data suggest that defective TC-NER, resulting from genetic abnormalities in the CS-B gene, particularly, in exons 2 and 5, may contribute to malignant progression in gliomas.

#2510 Y-Box Binding Protein-1 Binds Preferentially to Single-Stranded Nucleic Acid and Exhibits 3' - 5' Exonuclease Activity. Hiroto Izumi, Toshihiro Imamura, Gunji Nagatani, Tomoko Ise, Tadashi Murakami, Hidetaka Uramoto, Takayuki Torigoe, Hiroshi Ishiguchi, Yoichiro Yoshida, Minoru Nomoto, and Kimitoshi Kohno. *Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan, and University of Occupational and Environmental Health, Fukuoka, Japan.*

We previously have shown that YB-1 (Y-box binding protein-1) binds preferentially to cisplatin-modified Y-box sequences. Based on structural and biochemical data, we predicted that this protein binds single-stranded nucleic acids. In the present study we confirmed the prediction and also discovered some unexpected functional features of Y-box binding protein-1. We found that the cold-shock domain of the protein is necessary but not sufficient for double-stranded DNA binding while the C-tail domain interacts with both single-stranded DNA and RNA independently of the cold-shock domain. In an in vitro translation systems, the C-tail domain of the protein inhibited translation but the cold-shock domain did not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonucleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimerization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of the protein. The region between residues 51 and 205 of its 324-amino acid extent is required for full exonuclease activity. Our findings suggest that Y-box binding protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

3'-5' exonucleases are proteins that ensure the accuracy of DNA replication by catalyzing the removal of mispaired nucleotides from the 3'-termini of nascent DNA. Base mutations arising through nucleotide misinsertions are considered to be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α) lacks an intrinsic proofreading exonuclease, although it has been suggested that an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of either primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripheral blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exonuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown that exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides

EXHIBIT 7

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Lauren Schnaper, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Robert J. Hickey, Pamela E. Bechtel, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Linda H. Malkas, Derek J. Hoelz, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/7/06

Lauren A Schnaper

Lauren Schnaper

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

#2507 Detection of the Cancer Specific Form of PCNA by Elisa Assay. D. Tomic, D. J. Hoelz, P. Wills, R. J. Hickey, L. Schnaper, C. Lankford, and L. H. Malkas. *Greater Baltimore Medical Center, Towson, MD, and University of Maryland, Baltimore, MD.*

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which is required for DNA replication (processivity factor of DNA polymerase δ) and DNA repair. Previously, using 2D-IEF-PAGE analyses, our laboratory discovered that malignant breast cells express a unique, acidic form of PCNA protein which can clearly be distinguished from the basic form of this protein found in non-malignant cells. Our research suggests that the acidic form of PCNA is, most likely, the result of a post translational modification. This finding is important because this unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of this malignancy. Therefore, the purpose of this study was to develop an ELISA test, which can distinguish the malignant form of PCNA from the non-malignant form. We tested the hypothesis that xeroderma pigmentosum (XP)G protein, a structure-specific repair endonuclease similar to FEN1, and used in the nucleotide excision repair pathway is capable of distinguishing two forms of PCNA through binding affinities. To test this hypothesis, the protein isolated from the non-malignant breast cell line (MCF10A) and the breast cancer cell line (MCF-7) were used to measure the binding affinity of XPG to the acidic and basic form of PCNA in a modified ELISA assay. Standard curves, representing the correlation between absorbance and the abundance of the malignant and non-malignant form of PCNA were prepared and compared to each other. Serial dilutions of PCNA were tested in duplicate and the mean value of absorbance was calculated and used for comparison. Our results indicate that XPG protein has a different binding affinity for the malignant and non-malignant forms of PCNA. These results are the first to demonstrate that these two forms of PCNA can be distinguished by an ELISA assay that can be used clinically for the early detection of breast cancer.

#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
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We previously have shown that YB-1 (Y-box binding protein-1) binds prefer entially to cisplatin-modified Y-box sequences. Based on structural and biochem ical data, we predicted that this protein binds single-stranded nucleic acids. In the present study we confirmed the prediction and also discovered some unexpected functional features of Y-box binding protein-1. We found that the cold-shock domain of the protein is necessary but not sufficient for double-stranded DNA binding while the C-tail domain interacts with both single-stranded DNA and RNA independently of the cold-shock domain. In an in vitro translation systems, the C-tail domain of the protein inhibited translation but the cold-shock domain did not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonucleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimerization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of the protein. The region between residues 51 and 205 of its 324-amino acid extent is required for full exonuclease activity. Our findings suggest that Y-box binding protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

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EXHIBIT 8

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Derek J. Hoelz, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Robert J. Hickey, Pamela E. Bechtel, Lauren Schnaper, Min Park, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Linda H. Malkas, Lauren Schnaper, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8-6-06

Derek J. Hoelz

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

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We previously have shown that YB-1 (Y-box binding protein-1) binds preferentially to cisplatin-modified Y-box sequences. Based on structural and biochemical data, we predicted that this protein binds single-stranded nucleic acids. In the present study we confirmed the prediction and also discovered some unexpected functional features of Y-box binding protein-1. We found that the cold-shock domain of the protein is necessary but not sufficient for double-stranded DNA binding while the C-tail domain interacts with both single-stranded DNA and RNA independently of the cold-shock domain. In an in vitro translation systems, the C-tail domain of the protein inhibited translation but the cold-shock domain did not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonucleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimerization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of the protein. The region between residues 51 and 205 of its 324-amino acid extent is required for full exonuclease activity. Our findings suggest that Y-box binding protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

3'-5' exonucleases are proteins that ensure the accuracy of DNA replication by catalyzing the removal of mispaired nucleotides from the 3'-termini of nascent DNA. Base mutations arising through nucleotide misinsertions are considered to be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α) lacks an intrinsic proofreading exonuclease, although it has been suggested that an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of either primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripheral blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exonuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown that exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides

EXHIBIT 9

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Dragana Tomic, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Pamela E. Bechtel, Lauren Schnaper, Min Park, and Derek J. Hoelz, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Linda H. Malkas, Lauren Schnaper, and Derek J. Hoelz are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 08/16/08

Dragana Tomic

Dragana Tomic

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

#2507 Detection of the Cancer Specific Form of PCNA by Elisa Assay. D. Tomic, D. J. Hoelz, P. Wills, R. J. Hickey, L. Schnaper, C. Lankford, and L. H. Malkas. *Greater Baltimore Medical Center, Towson, MD, and University of Maryland, Baltimore, MD.*

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which is required for DNA replication (processivity factor of DNA polymerase δ) and DNA repair. Previously, using 2D-IEF-PAGE analyses, our laboratory discovered that malignant breast cells express a unique, acidic form of PCNA protein which can clearly be distinguished from the basic form of this protein found in non-malignant cells. Our research suggests that the acidic form of PCNA is, most likely, the result of a post translational modification. This finding is important because this unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of this malignancy. Therefore, the purpose of this study was to develop an ELISA test, which can distinguish the malignant form of PCNA from the non-malignant form. We tested the hypothesis that xeroderma pigmentosum (XP)G protein, a structure-specific repair endonuclease similar to FEN1, and used in the nucleotide excision repair pathway is capable of distinguishing two forms of PCNA through binding affinities. To test this hypothesis, the protein isolated from the non-malignant breast cell line (MCF10A) and the breast cancer cell line (MCF-7) were used to measure the binding affinity of XPG to the acidic and basic form of PCNA in a modified ELISA assay. Standard curves, representing the correlation between absorbance and the abundance of the malignant and non-malignant form of PCNA were prepared and compared to each other. Serial dilutions of PCNA were tested in duplicate and the mean value of absorbance was calculated and used for comparison. Our results indicate that XPG protein has a different binding affinity for the malignant and non-malignant forms of PCNA. These results are the first to demonstrate that these two forms of PCNA can be distinguished by an ELISA assay that can be used clinically for the early detection of breast cancer.

#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
occupancy show similar binding
ylguanine. We conclude, therefore, that while zinc is neither essential for DN repair by hAGT nor required for maintaining a functional fold of the protein, th presence of the transition metal ion bound within the polypeptide structur confers a mechanistic enhancement to repair activity which does not result fro an increase in substrate binding affinity. Zinc may also provide some measure o structural stability to hAGT.

#2509 Molecular Alterations in the Transcription-Coupled Nucleotide Excision Repair Gene, CS-B/ERCC6, in Human Malignant Gliomas. Franc Ali-Osman, Kurt Jaeckle, Thomas Connor, Gamil Antoun, and Lixin Zhan *U.T. M.D. Anderson Cancer Center, Houston, TX.*

The CS-B/ERCC6 gene encodes a complementation factor required for effi cient transcription-coupled nucleotide excision repair (TC-NER), a major DN repair pathway by which a variety of lesions are removed from the cellula genome. In this study, we examined 39 primary human malignant glioma speci mens and their matched normal tissues, as well as, 11 early passage glioma cel lines, for molecular alterations (deletions and mutations) in the CS-B gene. The results were correlated with the histological grade of the tumors. The result showed, overall, CS-B gene deletions to increase with increasing glioma grade and exon II to be most frequently deleted exon. Frequencies of exon II deletion were 12.5%, 30.8% and 66.7%, in astrocytomas, anaplastic astrocytomas and glioblastoma multiformes, respectively. Mutation analysis performed by SSC analysis of exon II and confirmed by nucleotide sequencing, showed a lowe frequency of mutations in exon II of the CS-B gene in the tumors, with only 12.5% of the glioma, all anaplastic astrocytoma or glioblastoma multiforme, to harbo any mutations. The mutations were varied and comprised of nucleotide trans tions of CAC(H)→TAC(Y) in codon 13 and CAA(C)→CGG(H) in codon 15, and transversions of TCT(S)→TAT(Y) and CAG(Q)→CAC(H) in codons 57 and 7 respectively. These data suggest that defective TC-NER, resulting from genetic abnormalities in the CS-B gene, particularly, in exons 2 and 5, may contribute to malignant progression in gliomas.

#2510 Y-Box Binding Protein-1 Binds Preferentially to Single-Stranded Nucleic Acid and Exhibits 3' - 5' Exonuclease Activity. Hiroto Izumi, Toshihir Imamura, Gunji Nagatani, Tomoko Ise, Tadashi Murakami, Hidetaka Uramoto, Takayuki Torigoe, Hiroshi Ishiguchi, Yoichiro Yoshida, Minoru Nomoto, and Kimi toshi Kohno. *Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan, and University of Occupational and Environmental Health, Fukuoka, Japan.*

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3'-5' exonucleases are proteins that ensure the accuracy of DNA replication by catalyzing the removal of mispaired nucleotides from the 3'-termini of nascent DNA. Base mutations arising through nucleotide misinsertions are considered to be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α) lacks an intrinsic proofreading exonuclease, although it has been suggested that an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of either primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripheral blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exonuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown that exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides